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PHYTOPATHOLOGICAL AND BOTANICAL RESEARCH METHODS

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BY

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PREFACE

In this book an attempt has been made to present methods which are of use to phytopathologists. Many of the methods are useful in other botanical sciences also.

Before preparing the manuscript, considerable work was done to develop new experimental methods and to improve technics described by other workers. The methods described are those which were found to be most satisfactory.

An extensive bibliography has been included. Many of the papers cited describe technics which have not been used by phytopathologists but which appear to be applicable in pathological work.

I am indebted to Professors R. E. Smith, M. W. Gardner, J. T. Barrett, and J. P. Bennett for reading portions of the manuscript and for suggesting improvements, and to Dr. William N. Takahashi, who prepared the illustrations and did part of the experimental work necessary in developing some of the methods.

T. E. RAWLINS.

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CHAPTER I

CHOOSING AND PLANNING A RESEARCH PROJECT

TENTATIVE SELECTION OF A RESEARCH PROJECT

It is seldom, if ever, advisable to make a final choice of a research project until a thorough study has been made of related literature. On the other hand, it is necessary that a tentative selection of the project be made in order that the volume of related literature to be reviewed may be within practical limits.

REVIEW OF LITERATURE RELATED TO PROPOSED RESEARCH

A thorough review of literature related to the proposed research is usually very helpful. Such a study sometimes reveals that the proposed work has already been done or may suggest various modifications of the proposed research.

Although some of the published papers on a particular disease or subject may be cited by text books of plant pathology, a further search often discloses other very valuable papers. Such a search may be most efficiently conducted by first selecting key words for the proposed research subject and then determining what literature is listed under these words in various indexes. The common and scientific names of the host plant and the casual organism, and the various names applied to the disease, are often good key words. The key words should be written in alphabetical order on a card and a search should then be made for them in each of the indexes. Following is a list of indexes and other papers which are very useful to the phytopathologist in locating literature related to his proposed research:

1. Oxford list of scientific periodicals.
2. Card catalogue of University Library.
3. Card catalogue of Agricultural Library.
4. Card catalogue of Botany Library.
5. Card catalogue of separates in Plant Pathology Library.
6. Agricultural Index.

7. Botanical Abstracts.
8. Oudemans, C. A. J. A. Enumeratio systematica fungorum. 1924.
9. Check list of diseases of economic plants in the United States. U. S. D. A. Bull. 1366, 1926.
10. Seymour, A. B. Host index of the fungi of North America. Harvard University Press, Cambridge, Mass. 1929.
11. Dorfler, I. Botaniker-Addressbuch. 1909.
12. Experiment Station Record.
13. International Catalogue of Scientific Literature—Botany.
14. Hollrung, M. Jahresbericht Ueber das Gebiete der Pflanzenkrankheiten.
15. Phytopathology.
16. Review of Applied Mycology.
17. Rivista di Patologia Vegetale.
18. Biologische Reichanstalt für Land und Forstwirtschaft. Bibliographie der pflanzenschuts Literatur.
19. Saccardo, P. A. Sylloge Fungorum.
    Vol. 12—Index to fungi.
    Vol. 13—Host index.
    Vol. 15—Synonymy.
    Vols. 19 and 20—Index to illustrations.
20. Journal of Agricultural Research.
21. U. S. D. A. Library Bibliographical Contributions 8 and 16.
22. Biological Abstracts.
23. Heald, F. D. Manual of Plant Diseases. McGraw-Hill Book Co., New York. 1926. (Gives list of text books on plant diseases, pp. 20–24.)
24. Chemical Abstracts.
25. Physiological Abstracts.
26. Abstracts of Bacteriology.
27. Index Medicus.

The index numbers of *Experiment Station Record, Review of Applied Mycology,* and *Biological Abstracts* are especially useful when searching for literature.

Each reference found should be listed at the top of a separate filing card.

The following form of literature citation has been found to be quite satisfactory for use on filing cards:

> Jones, F. R., and C. Drechsler. Crownwart of alfalfa caused by Urophlyctis alfalfae. Jour. Agr. Res. **20**: 285–323. 1920.

As a paper is being read it should be abstracted on the filing card. In reading a paper one should make a critical study of the methods used and the results obtained. These should be recorded on the card. The author's summary and conclusions should not be used as a card

record unless they appear to be warranted by the results. Sentences which summarize the results should be copied as quotations when possible. Such quotations may often be used when publishing a review of the literature.

When the available literature has been thoroughly studied a review of this literature should be written. Such a review should include the results obtained by previous workers, together with an attempt to explain any inconsistencies in the results and to interpret the combined results of all the workers. The preparation of such a review aids in clarifying the author's ideas on the subject and may therefore improve his qualifications for planning and carrying on related experimental work. This review should be saved for later use in publication.

FINAL SELECTION OF THE RESEARCH PROJECT AND PREPARATION OF PLAN OF EXPERIMENTS

After thoroughly reviewing the literature the worker should be able to define sharply the experimental work which he desires to undertake. However, before beginning experimental work it is usually desirable to make a detailed plan of the proposed research.

Since the results of most research are published, the work should be so planned that they may be clearly presented to a reader. When possible, it is desirable to present experimental results graphically or in tables, those types of presentation bringing the results together in forms which can be easily analyzed. In order to present results in these forms it is necessary that uniform methods be used in the experimental work. Otherwise the necessary explanatory notes become so involved, or the number of columns in the table so great, that the results are very difficult to interpret.

It is usually desirable to prepare blank tables for recording data before beginning experimental work. These tables should then be thoroughly studied as to possible results, sufficiency of data, control of various factors, reliability of data, possible errors, etc. Such a study often suggests various desirable modifications of the plan.

Whenever possible, the experiments should be on a large enough scale so that the results will be conclusive. Probably the most common cause of failure in phytopathological research is the tendency of workers to carry on what may be called "preliminary experiments." Such experiments are usually on such a small scale as to yield inconclusive results and therefore leave the worker's knowledge where it was before beginning the experiments.

As the work progresses, the results should be recorded in the tables. The recording of data from day to day in diary form should be avoided, as this tends to produce incomplete records which lack uniformity and are therefore very difficult to interpret or to present in a publication.

PART II

EXPERIMENTAL METHODS

Most of the experimental methods used by the plant pathologist may be included in one of three classes. The first class includes the various microscopic methods; the second class, the numerous culture and inoculation methods; the third, the methods used in virus studies. Miscellaneous methods, which do not fall in any of these classes, have been placed in a fourth group.

CHAPTER II

MICROSCOPIC METHODS

Because of the emphasis which has been given Koch's postulates, workers tend to adopt a routine of plating out diseased tissue without first making a thorough microscopic examination. The organisms isolated by the plating methods are later inoculated into healthy plants to determine whether they cause the disease. This routine frequently involves considerable work and loss of time in the study of secondary invaders. A thorough microscopic study of plants infected by several organisms may sometimes give evidence as to which is causing the disease, and may therefore prevent the unnecessary study of secondary invaders.

Plant pathologists usually employ unstained sections in diagnostic work. This method can be quickly applied and is often useful in detecting invading organisms. The use of staining methods is often desirable even though they require considerably more time than is required for preparing unstained preparations. The paraffin method is often necessary for demonstrating fine details and is especially desirable for preparing sections which are to be photographed.

FREE-HAND SECTIONS

Every plant pathologist should learn to cut satisfactory free-hand sections, for frequently this is the only available method in field laboratories, and if properly followed it may give sections which are as satisfactory as those prepared by using the freezing microtome, the hand microtome, or the sliding microtome. Either a safety razor blade or a sectioning razor may be used in free-hand work.

Pieces of material larger than 1 cm. in thickness may usually be held in the hand while cutting. Smaller pieces may be held in pith during sectioning. The pith may be kept in 50% alcohol until just before using, when it should be dipped in water for a moment to remove excess alcohol which would plasmolyze tissues. Fresh carrot

may be used instead of pith. The carrot tissue does not dull the razor as rapidly as does the pith. Most workers prefer to use dry pith, the razor also being kept dry while cutting. A dull razor should never be used in sectioning, since a dull edge can only produce thick or distorted sections. Sections should not be allowed to become dry but should be placed in water as soon as cut. In order that the sections may easily be seen, the water should be contained in a white dish or in a flat glass receptacle placed over a white paper. By means of a wire loop the sections may then be lifted from the water and transferred to a drop of liquid on a slide, or to a fixing solution contained in a vial.

Mycelium may frequently be detected by merely crushing infected tissues on a slide. Bacteria may readily be observed if the infected tissues are simply sliced and mounted in water, the bacteria soon moving out into the water adjacent to the cut edge.

USE OF THE HAND MICROTOME

The hand microtome may be used for cutting sections of fixed or unfixed woody material which is sufficiently hard to be held firmly by the clamps of the microtome. A safety razor blade, such as is used in free-hand sectioning, is unsatisfactory for use with this microtome. A sharp sectioning razor or a microtome knife with sharpening handle and sharpening back attached should be used with this apparatus.

The portion of the razor over which the sections slide, and the surface of the microtome on which the razor slides, should be kept flooded with some liquid having a low surface tension such as a 0.5% gelatin solution or a dilute soap solution. As the sections are cut they should be removed to distilled water by means of a moist camel's-hair brush. After being in water for 5 minutes to remove the soap or gelatin solution they may be placed on a slide for examination or transferred to a fixing solution.

The hand microtome is sometimes used also for succulent tissues. In such cases the material to be sectioned is held in pith as in free-hand sectioning, the base of the pith being held by the clamp of the microtome. A rubber band should be wrapped about the upper portion of the pith to hold the tissues in place, but should not be sufficiently tight to crush the tissues. Lime pith is said to be more rigid and therefore more suitable for this work than the ordinary elder pith. When difficulty is experienced in holding tissues in a firm position the

tissues may be surrounded by paraffin and the base of the paraffin attached to a solid block of wood.

THE SLIDING MICROTOME

The technic in using the sliding microtome is so similar to that for the hand microtome that it does not appear necessary to repeat it. In the case of the sliding microtome the knife moves on a slide and does not need to be held by the hand except during the sectioning stroke. In both cases the angle of the knife with the direction of movement of the knife should be as small as possible in order that there shall be maximum slicing and minimum pressure on the material being sectioned.

The adjustment for thickness of section should seldom be set for less than 15 microns, and often it is necessary to cut sections considerably thicker than this in order to prevent distortion of the sections by the cutting stroke.

MOUNTING UNSTAINED MYCELIUM OR SECTIONS

Aerial mycelium is often difficult to wet with water because of the high surface tension of the latter. Consequently, when such mycelium is mounted in water for microscopic examination, much of the mycelium is found to be surrounded by bubbles of air which interfere with its visibility. If the mycelium is first immersed in a solution having low tension, such as a 0.5% gelatin solution or a soap solution, these air bubbles are removed. The mycelium should then be immersed in water to remove the gelatin or soap and may then be mounted in water or lacto-phenol on a slide. Alcohol should not be used for removing air as it often causes distortion of the mycelium or attached spores. Dr. J. T. Barrett recommends 3% potassium hydroxide for wetting and swelling mycelium and spores. This solution is particularly useful for swelling dried specimens.

Unstained sections may be mounted in lacto-phenol if permanent preparations are desired. Place a small drop of lacto-phenol on the slide, warm over a flame and add the mycelium or sections by means of a wire loop. A cover glass is then added and is sealed with thick balsam. If too much lacto-phenol is used it may extend beyond the edge of the cover glass and thus interfere with sealing. Mounting in lactic acid is reported to prevent the detachment of conidia from conidiophores.

STAINING UNFIXED SECTIONS AND UNFIXED SUPERFICIAL MYCELIUM

Unfixed organisms seldom stain well. If the concentration of the staining solution is great enough to stain the structures in the cell sufficiently it usually kills the cell, and causes a collapse of the protoplasm. Very dilute solutions of certain non-toxic vital stains have sometimes been used for staining structures in living cells. See McClung (175). It is sometimes possible to increase the visibility of unfixed microorganisms by using one of the following staining methods.

RUTHENIUM RED METHOD

Make a 0.01% aqueous solution of the stain. Fill a staining dish to a depth of about 2 mm. with this solution. By means of a wire loop transfer the mycelium or sections from water to the staining solution and place the dish on the stage of a microscope so that the staining process may be watched. In the case of aerial mycelium it may be necessary to dip the mycelium in a 0.5% gelatin solution or a 1 to 10 dilution of liquid soap to remove air before placing the mycelium in the first water. When the sections or mycelium have been sufficiently stained they should be transferred to water for about 1 to 2 minutes to remove the excess stain, and may then be mounted in water on a slide for microscopic examination.

Unfixed sections or superficial mycelium may be stained on a slide as follows: Mount material in a drop of water on the slide, add cover glass, place a drop of ruthenium red solution at the edge of the cover glass, and draw water under the cover glass into filter paper by touching the filter paper to the water on the opposite side of the cover glass from the drop of staining solution. This procedure should be repeated until the staining solution has replaced the water under the cover glass. The staining may be watched through the microscope. Should the material become too heavily stained, some of the stain may be removed by replacing some of the staining solution with water in the same manner that the water was replaced by the staining solution. Dr. Lee Bonar has found this method satisfactory for demonstrating septa in spores.

Ruthenium red is very expensive and quite unstable. The staining solution should therefore be kept in the dark when not in use. Even when this precaution is observed the solution becomes unsatisfactory for staining after a few days.

COTTON BLUE METHOD

Unfixed mycelium may best be stained by mounting it in a ho 0.05% to 0.1% solution of cotton blue in lacto-phenol. If it is desire to keep the preparation for some time the cover glass should be seale with thick Canada balsam or melted vas-par. The cotton blue stain the protoplasm of the fungus but does not stain the cell walls. Con sequently, the septa of spores and mycelium stand out very distinctly Unfortunately, the entire protoplast is stained, the cytoplasm an nuclei being indistinguishable.

Lacto-phenol contains equal parts of phenol, lactic acid, glycerin and distilled water. Some workers use 2 parts of glycerin to 1 par of each of the other constituents.

Vas-par is made by mixing equal parts of melted paraffin an vaseline. It is placed along the edge of a cover glass by means of a warm glass rod or the warm melted material may be applied by mean of a fine-pointed camel's-hair brush.

FIXATION

In fixation an attempt is made to fix and harden the protoplasm so that it may retain its original structure during various later proc esses such as staining, dehydrating, imbedding, etc. If living cell are treated with toxic staining solutions the protoplasm loses it original structure and its more or less uniform distribution in the cel and accumulates in amorphous masses in various parts of the cell In this latter condition it has little resemblance to living protoplasm However, well-fixed protoplasm can be treated with such staining solutions or with dehydrating reagents without much visible chang in its structure.

The fixation process is apparently mainly a coagulation of th hydrophilic colloids of the protoplasm. Some of the reactions betwee proteins and compounds commonly used in fixing solutions are wel known. Chromic acid, picric acid, and formaldehyde are known t react with proteins to form insoluble compounds. Formaldehyde i thought to react with the free amino groups of the proteins to form insoluble compounds as follows:

$$-NH_2 + O = CH_2 \rightarrow -N = CH_2 + H_2O$$

According to Robertson (212), proteins are precipitated by alcoho in the form of salts. It is thought that upon the addition of alcoho

the ions of the protein salts become associated as uncharged molecules and are therefore precipitated.

A good fixing solution should coagulate the constituents of protoplasm as fine particles thus causing little change in its appearance, should produce a stable fixation that remains unchanged during later treatments, should not destroy any of the structures in the protoplasm, and should give the protoplasm satisfactory staining properties. Each fixing solution causes the protoplasm to assume a slightly different appearance and probably none of them produce exactly the appearance of living protoplasm.

Certain reagents commonly used in fixing solutions are known to remove some of the visible constituents of protoplasm by solution. For example, fats are removed by chloroform, and acetic acid dissolves mitochondria. Strangeways and Canti (236) studied the fixation of protoplasm by means of the darkfield microscope and found that a 2% solution of osmic acid produced the least change of any of the solutions tried.

Certain fixing solutions produce unfavorable staining properties in tissues. Formaldehyde is particularly active in reducing the avidity of tissues for stains. Chromic acid produces a fixation having particularly good staining qualities. If tissues which have been treated with formalin solutions are later treated with a solution of chromic acid the avidity of the tissues for stains is restored.

The length of time required for fixation varies with the material to be fixed and the solution used. We have found that certain material such as aerial mycelium is not sufficiently fixed by chromic acid fixing solutions after 24 hours and may collapse when placed in alcohol unless fixed for 48 hours or more. In general it appears best to fix for at least 48 hours unless experiments have shown that a shorter period is sufficient for the material to be studied.

After fixation the fixing solution should be thoroughly washed from the tissues. Any fixing solution remaining in the tissues is likely to prevent satisfactory staining.

PREPARING MATERIAL FOR FIXATION

Material which is dry and waxy and not easily wet by the fixing solution should be dipped in a dilute soap solution and then washed in distilled water before being placed in the fixing solution. In order that the fixing solution may penetrate quickly and in high concentration it is necessary that the tissues should be cut into small pieces.

When possible these pieces should be 5 mm. or less in thickness. The tissues should always be fresh and turgid when fixed. If material has wilted it should be placed in cold water until turgidity is restored.

FIXING SOLUTIONS

Following is a list of fixing solutions which we have found most satisfactory for diseased tissues:

Karpechenko's Solution

A. Chromic acid	1 g.
Acetic acid	10 cc.
Distilled water	90 cc.
B. Formalin	10 cc.
Distilled water	40 cc.

Equal parts of solutions *A* and *B* should be mixed just before using.

For general use, this appears to be the most satisfactory fixing solution that we have found. It penetrates well, causes little distortion, and produces a stable fixation. Material should be fixed in this solution for 48 hours and should then be washed in running water from 6 to 12 hours. If *sections* do not stain brightly after fixing in this solution they should be washed and then treated with a solution containing 0.3% chromic acid and 1.5% acetic acid for 15 minutes to an hour. Then wash in water for 30 minutes before staining.

Flemming's Weak Solution

2% osmic acid	2.5 cc.
1% chromic acid	12.5 cc.
1% acetic acid	5 cc.
Distilled water	30 cc.

Fix 48 hours. Wash in running water 6 to 12 hours. Osmic acid is reduced by organic matter and produces a blackening of the tissues. Sections should therefore be bleached in equal parts of commercial hydrogen peroxide and 95% alcohol for about 24 hours, and should then be washed in water for 30 minutes before staining.

This solution produces excellent fixation and is followed by very bright staining. Flemming's solutions penetrate slowly and should not be used for leaves or stems unless the tissues are cut into very small pieces.

Allen's Solution

Chromic acid	1 g
Acetic acid	1 cc.
Urea	0.5 g.
Distilled water	100 cc.

Fix 48 hours. Wash in running water 6 to 12 hours. This solution produces good fixation of certain tissues and is followed by bright staining. No bleaching is necessary after this solution.

Alcohol-Formalin-Acetic Solutions

	No. 1	*No. 2*
50% alcohol	100 cc.	100 cc.
Formalin	6.5 cc.	10 cc.
Acetic acid	2.5 cc.	10 cc.

Fix 48 hours or as long as desired. No washing is necessary. When ready to dehydrate, pass through 50% alcohol and higher grades. The fixing solution is washed from the tissues during this dehydration process.

Solution No. 1 causes plasmolysis in superficial mycelium and is therefore unsatisfactory for conidiophores or fungi grown in culture. Host tissues and mycelium within such tissues are well fixed by this solution. Tissues fixed and stored in it stain well.

Solution No. 2 is better for fungi which are not within host tissues. If material fixed in this solution does not stain well it should be treated with a chrom-acetic solution as after Karpechenko's solution.

Material fixed and preserved in either of the above alcohol-formalin-acetic solutions has a good consistency for free-hand sectioning.

Flemming's Strong Solution

1% chromic acid	75 cc.
Acetic acid	5 cc.
2% osmic acid	20 cc.

Numerous workers have used the above solution diluted with an equal volume of distilled water for the fixation of fungi.

Fixing Solutions for Mitochondria.—Mature plant cells usually contain large vacuoles and therefore plasmolyze more readily than meristematic cells which contain dense cytoplasm and small vacuoles. Acetic acid causes a swelling of protoplasm and thus tends to prevent plasmolysis. Most fixing solutions used by botanists therefore contain some acetic acid. The use of acetic acid is open to the objection that it destroys the mitochondria in the cells. Special fixing solutions

are therefore necessary for use in studying mitochondria. The following two solutions are recommended by Dufrénoy (78) for use in fixing diseased tissues:

Regaud's Solution

3% potassium bichromate	4 cc.
Formalin	1 cc.

Meves' Solution

2% osmic acid	1 cc.
1% chromic acid	5 cc.

Fix in either of these solutions 48 hours and wash in water 6 to 12 hours.

STAINING FIXED FREE-HAND SECTIONS

MAGDALA RED-LIGHT GREEN METHOD

By means of a wire loop, transfer sections from water to a fixing solution. After the sections have been in this solution for 15 minutes or longer, transfer them through five or six changes of water during a period of 20 minutes. The sections should then be placed in a fresh 0.25% solution of Grubler's magdala red in *tap* water for 1 minute to 24 hours, depending upon the avidity of the invading organism for the stain. If the organism shows little avidity for the stain the time required for staining may be decreased by increasing the concentration of the stain. The sections may be transferred to water at intervals and examined under the low power of the microscope to determine the progress of the staining. The staining should usually be considered sufficient when the invading organism becomes distinctly red. When sections are sufficiently stained, place them in tap water for 5 minutes to remove excess stain and then in absolute alcohol 30 seconds to dehydrate. Transfer sections to a 0.3% solution of Grubler's light green F.S. in clove oil until properly differentiated. The sections should be left in this stain until the host parenchyma tissues appear green when placed in clove oil and examined under the low power of the microscope. Place sections in clove oil 5 minutes or longer to remove excess light green. Mount in balsam.

The above method is a modification of that described by Dickson (70). This method is valuable in that it stains the fungus red and the host tissues green, thus clearly demonstrating the fungus and its distribution in the host tissues. Slides should be kept in the dark, as the light green fades quite rapidly.

Following is a brief schedule for the above method:

1. Cut sections.
2. Fixing solution, 15 minutes or longer (Flemming's weak solution).
3. Water, 20 minutes or longer.
4. Magdala red, 1 minute or longer.
5. Tap water, 5 minutes.
6. Absolute alcohol, 30 seconds.
7. Light green, until host parenchyma tissues are stained green.
8. Clove oil, 5 minutes.
9. Mount in balsam.

GENTIAN VIOLET METHOD

This method does not give differential staining. It is of use as a general histological stain because of its simplicity and its clear-cut staining. It is particularly useful in studying nematode galls.

After fixed sections have been washed through four or five changes of water, during a period of 15 to 20 minutes, transfer them to a 0.04% aqueous solution of Grubler's gentian violet for 25 to 45 minutes. Wash sections in water 5 minutes to remove excess stain, transfer to absolute alcohol for 10 to 30 seconds to dehydrate and to partially destain. The sections should then be transferred to clove oil. Clove oil slowly destains and differentiates the sections; they should be left in this liquid and watched through the microscope until they are properly differentiated. Mount in balsam.

Following is a schedule for this method:

1. Cut sections.
2. Fixing solution, 15 minutes or longer.
3. Water, 15 minutes to 20 minutes.
4. Gentian violet, 25 to 45 minutes.
5. Water, 5 minutes.
6. Absolute alcohol, 10 to 30 seconds.
7. Clove oil until destained.
8. Mount in balsam.

STOUGHTON'S METHOD (234)

This method gives excellent results on bacteria in tissues and is also used for staining fungi in tissues.

Following is a slightly modified schedule which we have found to be satisfactory for fire-blight:

1. Fix material in alcohol-formalin-acetic No. 1 for 48 hours or longer.
2. Cut *thin* hand sections or use sliding microtome for stem sections.
3. Wash sections in water ½ hour to 1 hour.

4. Stain in carbol-thionin 1 hour (1% solution of thionin in 5% solution of phenol).
5. Wash sections in water 10 to 15 minutes and then in absolute alcohol about 5 minutes.
6. Stain in orange G solution only long enough to stain parenchyma walls yellow (1 to 3 minutes). The orange G solution is prepared by mixing equal parts of a saturated solution of orange G in clove oil with a saturated solution of the same stain in absolute alcohol.
7. Place the section in clove oil about 3 to 5 minutes to remove the excess orange G. If the host walls are yellow and the parasite is dark in color after this treatment the section should be mounted in balsam. If the host walls are not sufficiently stained by the orange the section may be returned to the stain for a few seconds.

Paraffin sections are necessary to show the exact location of bacteria in tissues. They are scattered over the section during sectioning when free-hand methods or the sliding microtome are used.

JONES METHOD FOR GRAM-POSITIVE BACTERIA

Jones (130*a*) has obtained excellent results by applying the following staining method to tissues containing Gram-positive bacteria:

1. Fix tissues in alcohol-formalin-acetic solution No. 1.
2. Imbed in paraffin in usual way and section with microtome.
3. Stain in a dilute solution of safranin only long enough to stain the vessel walls red but not long enough to stain the walls of parenchymatous cells.
4. Wash in water for a few minutes.
5. Stain in gentian violet for 10 seconds or longer.
6. Place slide in iodine-potassium iodide solution about 10 seconds.
7. Dip slide in water.
8. Dip slide in absolute alcohol until stain ceases to run from sections.
9. Flood slide with a saturated solution of orange G in clove oil to which about 20% of absolute alcohol has been added. This solution stains the parenchyma walls yellow and also removes surplus gentian violet.
10. Clear with xylol and mount in balsam.

The bacteria are stained dark blue, the parenchyma walls yellow, and lignified walls red.

The gentian violet solution is prepared by mixing 10 cc. 95% alcohol and 2 cc. anilin oil. After shaking, 88 cc. of distilled water are added. Dissolve 5 g. of finely ground gentian violet in this mixture. Filter after solution occurs.

The iodine-potassium iodide solution is prepared by dissolving 2 g. potassium iodide and 1 g. iodine in 300 cc. distilled water.

CARTWRIGHT (35) METHOD FOR WOOD SECTIONS

This method appears to be the best of any that we have tried for staining infected wood. It should also be very useful in staining infected tracheæ or bast fibers of herbaceous plants. Both fungi and bacteria in lignified tissues are clearly demonstrated by the use of this method, the organisms staining blue and the lignified host cell walls staining red. Infected wood may be fixed either before or after sectioning. Alcohol-formalin-acetic solution No. 2 appears to be a very satisfactory fixing solution for wood.

Following is given a schedule which we have found to be satisfactory:

1. Cut fresh wood into small blocks about 1 cm. thick.
2. As soon as cut, fix blocks for 48 hours or longer.
3. Drop block into water for a few minutes before sectioning.
4. Cut sections and wash these in water for ½ hour.
5. Stain sections in a 1% aqueous solution of safranin about 1 minute.
6. Wash out excess stain in water.
7. Stain in picro-aniline blue. (To 25 cc. saturated aqueous solution of aniline blue add 100 cc. of a saturated aqueous solution of picric acid.) Cover section with stain and heat over a flame until just on the point of simmering.
8. Wash section in water.
9. Place section in absolute alcohol for about 30 seconds.
10. Clear in clove oil and mount in balsam.

If wood is found to be too hard to section well with a safety razor blade or a sliding microtome the wood may be placed in hydrofluoric acid after it has been thoroughly fixed and washed. It should be left in the hydrofluoric acid until the wood sections easily when tested with a safety razor blade. The material should be thoroughly washed before sectioning or staining to remove the hydrofluoric acid, which is very toxic to the skin. Do not inhale the fumes from this acid. The glycerin-thymol solution described later for dehydration may also be used as a softening solution for wood to be sectioned by free-hand or sliding microtome methods. A reagent called "diaphanol" and sold by E. Leitz Inc. has been reported by some to be useful for softening hard tissues.

Material which has been fixed and preserved in either of the alcohol-formalin-acetic fixing solutions may be sectioned, and after the sections have been washed for 15 to 20 minutes they may be stained by any of the above methods. If material has been fixed in other fixing solutions it should be washed in running water 6 to 12

hours and should then be run up through the alcohols to 70% alcohol in order to harden it sufficiently for sectioning. It may be stored in the 70% alcohol as long as desired.

The above staining methods may also be used for sections which have been cut by the use of the freezing microtome, the hand microtome, or the sliding microtome.

STAINING PARASITES IN UNSECTIONED TISSUES

Kohl (142*a*) reports that the distribution of a fungus on or within leaf tissues may be observed by fixing the tissues 24 hours in equal parts of 95% alcohol and glacial acetic acid and then clearing in a saturated solution of chloral hydrate. The tissues are then washed in water and the fungus is stained with dilute cotton blue or aniline blue. Glycerin is used as a mounting medium.

Godfrey (828) demonstrates nematodes in unsectioned roots by fixing in Flemming's solution until the nematodes are stained black. The roots are then dehydrated in alcohol and cleared in clove oil. The same technic may be used for locating insects or insect eggs in unsectioned tissues or in thick sections. The Flemming's solution must be sufficiently dilute to stain only the parasites and not the host tissues. The time of fixation should not be longer than is necessary to stain the parasites.

Dr. J. T. Barrett clears infected tissues by placing them in 75% lactic acid for several days. Colored mycelium, spores, or fruiting bodies may be readily seen in the cleared host tissues. If the fungus shows no color after clearing, the tissues should be mounted in lactophenol containing 0.05% to 0.1% cotton blue to stain the fungus.

PARAFFIN METHOD

FIXATION

Material to be imbedded in paraffin should be fixed as described under fixing solutions.

WASHING

After fixation the material should be placed in small cheesecloth sacks and should then be allowed to remain in running water for 6 to 12 hours. Tissues which have been fixed in alcohol-formalin-acetic solutions do not require washing, the fixing solution being removed by the alcohol during dehydration.

DEHYDRATION

After washing, it is necessary that the water in the material be replaced by some liquid which is miscible with water and is also miscible with a paraffin solvent. Ethyl alcohol has these properties and is therefore most commonly used for dehydration. It is generally considered that a direct change of material from water to alcohol causes plasmolysis and distortion of the cells. Material is therefore carried through a gradual series of increasing concentrations of alcohol to absolute alcohol, thus gradually replacing all the water in the material with alcohol. Following is given a dehydration schedule which has been found satisfactory for most material:

15% alcohol	10 to 20 minutes
30% alcohol	30 to 60 minutes
50% alcohol	1 to 2 hours
70% alcohol	3 to 24 hours
80% alcohol	6 to 24 hours
95% alcohol	1 to 3 hours
Absolute alcohol	2 changes ½ hour to 1 hour in each change

If it is inconvenient to complete the entire process according to the schedule given, the material may be stored in 70% alcohol until the later steps in the schedule can be completed.

Bradbury (25) has reported isopropyl alcohol to be superior to ethyl alcohol for dehydration. The tissues are said to be less brittle when the isopropyl alcohol is used.

We have found the following dehydration method to cause less distortion than the use of a graded series of ethyl alcohol concentrations. After washing, the material is placed in 10% glycerin in a large watch glass or similar container so that all the water will evaporate from the glycerin solution after 24 hours or longer. The evaporation may be hastened and the dust prevented from falling into the liquid by covering the container with filter paper which is allowed to come in contact with the glycerin solution. When the glycerin solution has assumed approximately the consistency of pure glycerin the material is washed in three changes of 95% alcohol during 3 hours until all the glycerin is removed. It is then transferred through two changes of absolute alcohol during 1 to 2 hours to complete the dehydration.

The addition of 1 cc. of a 10% solution of thymol in 95% alcohol to each 100 cc. of 10% glycerin prevents the growth of fungi in the glycerin solution.

INFILTRATION WITH A PARAFFIN SOLVENT AND WITH PARAFFIN

Since paraffin is not soluble in alcohol the alcohol in the dehydrated plant cells must be replaced by a paraffin solvent before the cells can be infiltrated with paraffin. Chloroform, xylol, benzol, and cedar oil are the paraffin solvents most commonly used. We have found benzol and cedar oil to cause the least distortion of tissues. The following schedule has been found to be satisfactory for infiltration of dehydrated material with benzol:

Solution of ¾ absolute alcohol and ¼ benzol ½ hour to 2 hours
Solution of ½ absolute alcohol and ½ benzol ½ hour to 2 hours
Solution of ¼ absolute alcohol and ¾ benzol ½ hour to 2 hours
Benzol—2 changes, ½ hour to 2 hours in each change.

Paraffin is dissolved in the paraffin solvent and the solvent is then gradually replaced by melted paraffin until the plant material is thoroughly infiltrated with it. The following infiltration schedule has been found to be satisfactory for most material: Add paraffin shavings to the benzol containing the plant material, leave the cork out of the vial, and place the open vial in a sparkless paraffin oven for about 24 hours. Pour off the paraffin-benzol solution and add melted paraffin. Change the paraffin two more times at approximately 24-hour intervals and then allow the material to remain in the oven for 48 hours after the last change.

Cedar oil produces less hardening of the tissues than benzol and is therefore preferable for lignified tissues. Dr. Ruth Allen has furnished the following schedule for the use of cedar oil:

33% cedar oil in absolute alcohol	4–24 hours
66% cedar oil in absolute alcohol	6 hours or longer
Pure cedar oil	6 hours or longer
Fresh pure cedar oil	24 hours or longer
25% paraffin in cedar oil (in oven)	4–24 hours
50% paraffin in cedar oil (in oven)	4–24 hours
75% paraffin in cedar oil (in oven)	4–24 hours
Melted paraffin	12–24 hours
Fresh melted paraffin	12–36 hours
Imbed in fresh paraffin	

IMBEDDING

Pour melted paraffin containing infiltrated plant material into a small box made of thin glazed cardboard. This box should be placed

well above a low flame or on a hot plate to prevent the paraffin from solidifying before the pieces of plant material are properly arranged in the paraffin. With a warm needle arrange the pieces of plant material in rows so that they will be in the proper position for sectioning. The paraffin should not be heated more than enough to keep it melted during the above operations, as a high temperature injures the tissues.

When the material has been arranged in the paraffin, remove the flame and bring a dish of cold water up under the paper tray until the surface of the water touches the bottom of the tray. As soon as the paraffin begins to solidify around the pieces of tissue, blow gently on the surface of the paraffin until a surface film is formed. Then carry the tray to a dish of cold waste alcohol and slowly force it under the alcohol. The cold alcohol aids in producing a homogeneous structure in the paraffin. (*Do not place the alcohol near a flame.*)

Material does not deteriorate in paraffin and may be kept in this condition as long as desired.

COMPLETE SCHEDULES FOR PARAFFIN METHOD

Following is a schedule which is usually satisfactory for the paraffin method. For convenience this will be called the standard schedule.

1. Fix in Karpechenko's solution 48 hours.
2. Wash in running water 6 to 12 hours.
3. Place in 10% glycerin and allow water to evaporate.
4. 95% alcohol, 3 changes during 3 hours.
5. Absolute alcohol, 2 changes during 1 to 2 hours.
6. ¼ benzol and ¾ absolute alcohol ½ to 2 hours.
7. ½ benzol and ½ absolute alcohol ½ to 2 hours.
8. ¾ benzol and ¼ absolute alcohol ½ to 2 hours.
9. Benzol, 2 changes during 1 to 4 hours.
10. Add paraffin shavings and place open vial in oven for about 24 hours.
11. Pour off benzol-paraffin mixture and add melted paraffin.
12. After approximately 24 hours pour off melted paraffin and add new melted paraffin.
13. After 24 hours again renew melted paraffin.
14. Imbed after 48 hours.

Zirkle (252) and others have recommended the use of normal butyl alcohol as a dehydrating agent and a paraffin solvent. The method has been found to be especially good for lignified tissues since the butyl alcohol causes little hardening of these tissues. Following is a schedule to follow fixation and washing:

Water	Ethyl alcohol	Normal butyl alcohol	Period of treatment
95	5	0	1 hour
89	11	0	1 hour
82	18	0	1 hour
70	30	0	1 hour
50	40	10	1 hour
30	50	20	Overnight
15	50	35	1 hour
5	40	55	1 hour
0	25	75	1 hour
0	0	100	1 hour
0	0	100	15–20 hours

Add paraffin shavings to the butyl alcohol containing the tissues and place in the paraffin oven. The later steps in the process are the same as in the standard schedule.

Hemenway (108) has found the following modification of the butyl alcohol schedule to be satisfactory and simple:

1. After fixing and washing, dehydrate with glycerin, using the method we have described under "dehydration."
2. After removing tissues from glycerin, transfer them to equal parts of glycerin and normal butyl alcohol for 36 hours.
3. Pure normal butyl alcohol, 36 hours in each of 2 changes.
4. Add paraffin shavings to the butyl alcohol and complete the process as in the standard schedule.

We have found the above schedule to give excellent results.

Kisser and Anderson (142) and numerous other workers have recommended the use of a combination of the celloidin method and the paraffin method for woody material.

SHARPENING THE MICROTOME KNIFE

Attach to the knife the handle and back provided for sharpening, and rub the knife gently on a fine-grained stone which is kept covered with water. The knife should always be moved with the edge forward and at an angle of about 45° with the direction of movement. Upon reaching the end of the stone the knife is turned over and the opposite edge is ground on the return stroke. A knife has usually been ground sufficiently when it appears fairly smooth when viewed under

the low power of the microscope. The knife should then be stropped for a short time to smooth the edge further.

ADJUSTING KNIFE IN MICROTOME

The angle at which the knife is held in the microtome is very important in cutting paraffin sections. If the knife is too nearly vertical the tissues will be crushed without being cut. Friction between the knife and the paraffin block is very noticeable when the knife is in such a position. On the other hand, if the knife is too nearly horizontal the sections will be scraped rather than cut. This maladjustment is usually accompanied by considerable noise as the sections are scraped from the block.

The knife may be most easily adjusted by placing it in the microtome at approximately the proper angle, placing the back used in sharpening on one end of the knife, and suspending a plumb-bob on the inner side of the knife. When the string is touching the cutting edge of the knife the lower portion of the string should be about 1 mm. from the sharpening back. This method, of course, can be used successfully only when the microtome is on a level table and when the sharpening back is the same size as that used in sharpening the knife.

After the position of the knife has been properly adjusted the screws for holding the knife in the microtome should be tightened sufficiently to hold it very firmly.

ATTACHING PARAFFIN BLOCK TO HOLDER

Paraffin is melted on the end of the holder and while this paraffin is still melted the block containing the material to be sectioned is fastened to the holder by placing it in the melted paraffin and then immersing in cold water. The union between the block and holder may be strengthened by adding more melted paraffin to the periphery of the union. The surface to be cut should be square or rectangular, and its upper and lower edges should be exactly horizontal when the block is attached to the microtome. The lot number and tissue block number should be scratched on the side of the block of paraffin to preserve its identity.

CUTTING SECTIONS

Adjust the microtome for cutting sections the desired thickness, and proceed with the cutting. If the sections fail to hold together to form a ribbon the paraffin is probably too cold or the sections are

too thick. The trouble may usually be remedied by increasing the temperature of the paraffin or cutting thinner sections.

In the case of woody material the sliding microtome or the sliding attachment on the rotary microtome should be used. The knife is turned to an angle of about 45° with the direction of movement of the block so as to give a slicing action as the knife edge passes through the tissue. Hemenway (108) floods the surface of the knife with a solution containing equal parts of glycerin and 95% alcohol. As the section slides off on this solution the edge may be held down with a camel's-hair brush to prevent curling. Each section is left on the knife for a few seconds and is then transferred to a dish containing the glycerin-alcohol solution. Kisser and Anderson (142) use a 0.5% solution of gelatin for flooding the knife to prevent curling. The sections are then attached to a slide as described under "Attaching Sections to Slide." We have found the gelatin solution to be most satisfactory.

If the imbedded tissue is found to be too hard to cut well, Conant recommends cutting off the end of the tissue and then immersing the paraffin block in water until cutting is improved. The water apparently passes into the tissues through the exposed surface. Couch (57) has modified this procedure by placing the block in 95% alcohol at 30°C. for 4 days to 3 weeks. The material is then placed in water 2 to 24 hours. A little carbol-fuchsin is used in both the alcohol and water to determine the rapidity of penetration.

ATTACHING SECTIONS TO SLIDE

With a blue wax pencil write lot number, tissue block number, and thickness of sections at one end of a clean slide. The wax of the pencil adheres better if the slide is slightly heated.

Place a very small drop of modified Szombathy's fixative on the slide and spread it to form a thin film. Add a drop of 2% formalin solution and float a portion of the paraffin ribbon on the solution. The slide is then slowly warmed over a flame or a slide warmer until the ribbon has become flat. The formalin solution is then withdrawn from the slide by absorbing it on the edge of filter paper, and the slide is allowed to dry for 2 or more days, care being taken to store it where dust will not settle on the sections.

Following is the formula for modified Szombathy's fixative:

Gelatin	1 g.
Glycerin	15 cc.
Water	100 cc.
Thymol (10% in 95% alcohol)	1 cc.

The gelatin is dissolved by heating in the water. The solution is then cooled and the glycerin and thymol are added in the order named. The solution is then filtered through cheesecloth. If a precipitate forms after standing for several months it may be dissolved by placing the bottle of fixative in warm water.

PREPARING SECTIONS FOR STAINING

The slides are now passed through xylol, alcohol, and water to remove the paraffin and to infiltrate the sections with water so that they can be stained. The liquids are kept in Coplin staining jars, and the slides are changed from one liquid to another according to the following schedude:

First xylol	5 minutes
Second xylol	5 minutes
Absolute alcohol	5 minutes
95% alcohol	5 minutes
Tap water, 3 changes during	10 minutes
Distilled water	2 minutes, if material is to be stained in iron alum-hematoxylin. Tap water causes an undesirable precipitate when introduced into iron alum solution.

BLEACHING

Sections which have been blackened by fixation in fixing solutions containing osmic acid should be bleached before staining. This is accomplished by placing the slides in a Coplin jar containing equal parts of commercial hydrogen peroxide and 95% alcohol for about 24 hours. The sections should then be washed in running water for about 30 minutes before staining.

STAINING

Various modifications of Heidenhain's iron alum-hematoxylin stain and Flemming's triple stain are usually most successful for plant material. Hematoxylin has the advantage of giving sharper definition, of photographing better, and of being more permanent than the triple stain. The latter has the advantage of being a differential stain, imparting different colors to different parts of the cell.

C. E. Allen's (3) schedules, with slight modifications, have been found to be very satisfactory. These schedules are as follows:

IRON ALUM-HEMATOXYLIN SCHEDULE

Liquid	Time	Concentration	Method of Application
Ferric ammonium sulfate solution.	2–24 hours.	2% in distilled water.	Coplin staining jar.
Distilled water	1–2 minutes		Coplin staining jar
Distilled water	2–5 minutes		Coplin staining jar
Distilled water	5–10 minutes		Coplin staining jar
Ripened solution of hematoxylin	20–60 minutes or longer	2% hematoxylin in 40% alcohol. Solution should stand a month or longer before using	Coplin staining jar
Tap water	1–2 minutes	*p*H must be high enough to produce blue color in sections	Coplin staining jar
Tap water	2–5 minutes	*p*H must be high enough to produce blue color in sections	Coplin staining jar
Tap water	5–10 minutes	*p*H must be high enough to produce blue color in sections	Coplin staining jar
Distilled water	5–10 minutes		Coplin staining jar
Ferric ammonium sulfate solution	Until sections are sufficiently destained	0 5% solution in distilled water	Examine sections at frequent intervals with microscope to observe destaining
Distilled water	5 minutes in each of 2 changes		Coplin staining jar
Running tap water	½-1 hour		Any suitable vessel
Absolute alcohol	1 minute		Pour over the slide from dropper bottle
Clove oil	5 minutes or longer		Pour over the sections from a dropper bottle and allow it to remain until sections are cleared. Pour off clove oil and absorb remaining clove oil around the sections on filter paper.
Mount in balsam			Remove clove oil from around the balsam by absorption on filter paper before adding cover glass.

A number of counterstains to follow the iron alum-hematoxylin have been recommended. Congo red, benzo-purpurin, orange G, light green F. S., and erythrosin are among those which have been most commonly used. Light green F. S. and erythrosin are usually dissolved in clove oil. We have usually found counterstains undesirable, best results being obtained with the hematoxylin schedule which we have given.

Schedule for Triple Stain

1. Prepare paraffin sections for staining according to method given under "Preparing Sections for Staining."
2. Stain in safranin solution ½ to 10 minutes.
3. Wash through 4 or 5 changes of tap water during 5 minutes.
4. Stain in gentian violet solution 3 to 20 minutes.
5. Wash through 4 or 5 changes of tap water during 5 minutes.
6. Stain with orange G solution 10 seconds or longer.
7. Dehydrate with absolute alcohol 10 to 30 seconds.
8. Destain with clove oil until properly differentiated.
9. Remove clove oil and mount in neutral balsam.

EXPLANATORY: The safranin and gentian violet solutions are kept in Coplin staining jars and the slides are placed in the solutions. The orange G, absolute alcohol, and clove oil are kept in dropper bottles and are poured over the sections on the slide according to the above schedule.

Different tissues stain very differently, and a given tissue fixed in dissimilar fixing solutions may stain quite differently. Therefore, it is necessary to experiment on each lot of material to be stained before an exact schedule can be given. In a cell containing a resting nucleus the nucleolus should stain red, the remainder of the nucleus a dark blue, and the cytoplasm light blue to orange. The period of staining in the different stains should be varied until this color combination is produced.

The destaining in clove oil may be watched under the microscope. Clove oil apparently has a differential destaining action, removing the gentian violet more rapidly from some structures than from others and thus producing very desirable differential staining. When the sections are properly destained the clove oil should be poured off, and as much of the remaining clove oil as possible should be absorbed on cloth or filter paper. About 2 drops of neutral Canada balsam dissolved in xylol are then added, and the clove oil displaced by the balsam is then absorbed on cloth or filter paper. Balsam hav-

ing an acid reaction should never be used since it causes stained preparations to fade.

The cover glass should be passed through a flame several times before it is placed over the sections. This heating appears to reduce the number of air bubbles remaining in the balsam under the cover glass.

Staining Solutions for Triple Stain

SAFRANIN: A 3% solution of water soluble or alcohol soluble safranin in a 1.7% solution of anilin oil in 45% ethyl alcohol.

GENTIAN VIOLET: A 2% solution in tap water.

ORANGE G: A 0.05% solution in distilled water.

The safranin and gentian violet made by different chemical companies vary considerably in staining properties. It may therefore be necessary to change the staining schedule or concentration of stains according to the source of the stains which are used.

Stains for Differentiating Host and Parasite.—The modified magdala red-light green method of Dickson (70) or the method of Stoughton (234) may be used for fungi or bacteria in unlignified tissues. Cartwright's (35) method should be tried for bacteria or fungi in lignified tissues. These methods have been described under "Staining Fixed Free-hand Sections," but are also applicable to paraffin sections. Other methods for the differential staining of host and fungus parasite are described by Vaughan (239), Osborn (191), Hubert (120), Durand (80), Gerry and Diemer (88), Lepik (155), and Ridgeway (211). Novy (188) and Wright and Skoric (247) have described other methods for staining bacteria in sections.

Staining methods which produce a distinct differential staining of host and parasite have seldom been found satisfactory for demonstrating the detailed protoplasmic structures of either. Therefore, if it is desired to study the smaller structures in either the host or parasite, most workers use the iron alum-hematoxylin method or the triple stain.

COTTON BLUE METHOD FOR DELICATE MATERIAL

Certain materials such as mycelium from cultures or aerial conidiophores on sections are very difficult to run up into paraffin or to mount in balsam because of the severe distortion which results during dehydration and clearing. The cotton blue method is very useful for

such material, the protoplasmic structures being fairly clearly stained and the material then being mounted with little distortion. The method also has the advantage of being very rapid.

Alcohol-formalin-acetic solution No. 2 has been found to be a very good fixing solution for use in this method. In the case of sections, the material is fixed either before or after free-hand sectioning. If fixed before sectioning the material should be fixed 48 hours or longer. If fixed after sectioning 20 minutes may suffice. The following schedule has been found to be satisfactory:

1. Fix sections or mycelium 20 minutes or longer.
2. Wash in water 20 minutes or longer.
3. Stain 10 to 15 minutes in the following solution and then mount in a drop of the same solution.

Phenol	10 g.
Glycerin	10 cc.
Lactic acid	10 cc.
Distilled water	10 cc.
Cotton blue	0.02 to 0.05 g.

4. Seal with thick xylol-balsam or vas-par.

If the above method does not give sufficient detail the material may be fixed and washed, and then stained with iron alum-hematoxylin. It is then subjected to McClung's (175) balsam infiltration mounting method. (The hematoxylin solution should not contain more than 10% alcohol. Higher concentrations of alcohol may distort the cells.)

STAINING MYCELIUM OR GERMINATING SPORES

The following method has been found to be very satisfactory for demonstrating the structure of the nuclei and cytoplasm in mycelium, the germ tubes of germinating spores, and in transparent spores. It may also be used in studying bacterial colonies.

Potato-dextrose-peptone agar is liquefied by heating, and a small drop of the liquid agar is placed on a warm slide. The edge of another slide is then quickly drawn across the agar, thus spreading it as a thin film on the first slide. As soon as the agar film has solidified it is dried by holding well above a low flame. A drop of a water suspension of mycelium or spores is spread on the agar film and the slide is placed in a moist chamber until the desired amount of mycelium growth has occurred. The slide is then quickly placed in Flemming's weak fixing solution for about 15 minutes, after

which it is washed in running water during a period of 30 minutes. It is bleached in equal parts of H_2O_2 and 95% alcohol for 24 hours and then washed for 30 minutes in running water. The slides are then stained with triple stain, or better, iron alum-hematoxylin and mounted in balsam according to the schedules given for staining paraffin sections.

Lutman (163) found the following method to be satisfactory for staining germinating smut spores. The spores are germinated in a liquid medium. A slide is smeared with albumin fixative and a few drops of the culture containing the germinated spores are drawn up in a pipette and placed on the slide. Flemming's weak fixing solution is then added. This fixes the germinating spores and coagulates the albumin, thus causing the spores to be held by the albumin. Part of the liquid on the slide is then allowed to evaporate. After washing, and bleaching in H_2O_2, stain with triple stain or hematoxylin.

Dodge and Gasier (74) find another method to be satisfactory for the study of germinating spores. The spores are germinated on agar in a Petri dish. Melted agar sufficiently cooled so that it will not injure the germ tubes is poured over the germinating spores and allowed to harden. The solid agar containing the germinating spores is then cut into blocks, is fixed, carried through the paraffin process, sectioned, and stained. This method is also reported to have been used for fungi growing on agar media and for diseased tissues from which the spores may be lost if left uncovered during the paraffin process. In the case of diseased tissues the material is dropped into clear liquid agar which has been cooled to 45° C. After gelation of the agar has occurred the agar containing the diseased tissues is cut into blocks as with germinating spores. Reagents penetrate agar very slowly. The agar blocks must therefore be left in each reagent longer than in the regular schedule.

Sass (214) also described a method for studying mycelium and germinating spores.

BURRI'S METHOD FOR DEMONSTRATING SMALL ORGANISMS

This method is useful for demonstrating small organisms which are not easily stained or for rapid work in which it is undesirable to spend the time required for staining the organisms. Fungus spores, bacteria, yeasts, and various other organisms may be demonstrated by this method. The principle of the method is quite the opposite of that in ordinary staining. The Burri method produces a dark field

which absorbs the light, but allows the light to pass through the organisms, whereas in ordinary staining methods the stained organisms absorb the light and the light passes through the surrounding field.

A small drop of a liquid medium containing the organism and a small drop of India ink are placed on the slide. The two drops are then mixed by means of a wire loop and the edge of a second slide is drawn across the liquid in order to spread it as a thin film on the surface of the first slide. As soon as the film becomes dry, Canada balsam and a cover glass are added. Some India inks are not satisfactory because they contain organisms or large particles of solid matter. Gunther and Wagner's "Chin Chin Liquid Pearl Ink" has been found to be satisfactory.

THEORY OF STAINING

Most dyes may be classed as basic or acid. Basic dyes form salts with acids; acid dyes form salts with bases. Most of our commonly used dyes are salts formed in this manner. For example, safranin O is a mixture of the following two compounds which may be regarded as salts formed by the reaction between color-bases and HCl.

$$C_{20}H_{19}N_4Cl$$
$$C_{21}H_{21}N_4Cl$$

Orange G on the other hand has the following formula, and may be regarded as a salt formed by the reaction between a color-acid and NaOH:

$$C_{16}H_{10}N_2O_7S_2Na_2$$

Such dye salts dissociate, the basic dye salts in safranin O dissociating into positively charged dye ions and negatively charged chloride ions while the acid dye salt orange G dissociates to form negatively charged dye ions and positively charged sodium ions.

Various theories have been advanced in an attempt to explain the chemistry of staining and dyeing. Perhaps the majority of those who have studied the staining process are inclined to the view that most staining of protoplasmic structure is due to the combination of basic dye cations and protein anions, or of acid dye anions and protein cations to form insoluble dye-protein compounds. According to this theory the *p*H of the staining solution should be very important in

staining since a protein molecule may become a cation at a *p*H below its isoelectric point or may be transformed to an anion at a *p*H above its isoelectric point. Thus it might be expected that a basic dye would stain a protein when in a solution having a *p*H above the isoelectric point of the protein whereas an acid dye should stain the protein when in a solution having a *p*H below the isoelectric point of the protein. Chapman, Greenberg, and Schmidt (38*a*) have studied the combination between pure proteins and dyes and have found that the above relation holds in most cases, the dyes and proteins combining in stoichiometric proportions and usually forming insoluble protein-dye compounds.

Naylor (183) studied the staining of root tips by acid and basic dyes at different hydrogen ion concentrations and found that the protoplasmic structures stained like proteins having an isoelectric region near *p*H 5. The chromosomes and nucleoli were found to retain the acid dyes at higher *p*H than the cytoplasm. This was interpreted as an indication that the chromosomes and nucleoli have a higher isoelectric point than the cytoplasm. When a mixture of acid and basic dye was used it was found that the acid dye stained below *p*H 4.6, both dyes stained between *p*H 4.6 and 5, and the basic dye stained above *p*H 5. Thus differential staining was obtained between *p*H 4.6 and 5.

Stearn and Stearn (230) report that Gram-positive bacteria usually stain as though they have an isoelectric point below *p*H 3, whereas Gram-negative bacteria usually stain as though they have an isoelectric point between *p*H 5 and 5.5.

Lee (153) reports that pure cellulose is not stained by the acid dye Congo red unless some salt such as sodium chloride is present. The same behavior is shown by charcoal, silk, alumina, silica, and other substances. This phenomenon is explained by Lee as follows: Filter paper (cellulose) has a negative charge when in suspension in water. Similarly the acid dye itself has a negative charge. Consequently, there is little tendency for the paper to adsorb the dye. When the ions of such a neutral salt as sodium chloride are present the sodium cation may be adsorbed by the paper, thus decreasing or annulling its negative charge until there is little or no obstacle to the adsorption of the dye. Basic dyes, on the other hand, stain paper more deeply, the lower the concentration of salt present. This is explained as being due to the paper being most highly charged in the absence of the salt and therefore having a greater tendency to adsorb the positively charged dye cation.

MICROCHEMISTRY

Microchemistry includes several fairly distinct fields. Quantitative work such as that developed by Pregl (419) constitutes one field of microchemistry. The identification of crystals by the use of the polarizing microscope is a second field. A third field includes the identification of compounds under the microscope by their reactions or solubilities in various reagents. We shall deal only with the latter two fields, from which methods have been chosen which may be applied in phytopathological work.

These methods may be applied in several ways. For example, organisms may be grown in synthetic culture media and the resulting compounds identified. Pathogenes or normal and diseased host tissues may be extracted with various solvents and the dissolved substances may be identified. Compounds may also be identified in the cells of pathogene or host.

IDENTIFICATION OF CRYSTALS

Space does not permit the explanation of the polarizing microscope or the optical properties of crystals. For information on these subjects the reader is referred to Chamot and Mason (281) and Winchell (472).

Before attempting to identify crystals it is desirable that a series of immersion liquids having different refractive indices be prepared. Directions for preparing such liquids are given by Chamot and Mason (281) and Winchell (472). After preparation the refractive index of each liquid is checked by means of an Abbe refractometer.

Mount the crystals in a liquid of medium refractive index. Cross the Nicol prisms and examine the crystals by means of the polarizing microscope. If a crystal is dark in all positions during the rotation of the stage it is isotropic and has only one refractive index which does not vary with the direction of transmission or direction of vibration of the light which passes through it.

To identify an isotropic crystal remove the analyzer and cover about two-thirds of the flat microscope mirror with a black card or paper. If the crystal has a lower refractive index than the mounting medium a shadow appears on the same side of the crystal as the exposed side of the mirror. For example, if the right third of the microscope mirror is left uncovered the right edge of the crystal appears dark and the left edge of the crystal is bright. If the crystal

has a higher refractive index than the mounting medium the left edge of the crystal appears dark and the right edge is bright. If the crystal has the same refractive index as the mounting liquid it is invisible or shows blue or red colors along the edge of the crystals. When the refractive index of the crystal has been determined this index is looked up in tables such as those given by Winchell (472*a*) for inorganic compounds or those in Vols. 1 and 7 of the International Critical Tables for inorganic and organic compounds. If a compound is found in the tables which has the determined refractive index and has other properties such as crystal shape, solubility, and melting point the same as the crystal under investigation the identification may be considered complete. Any characteristic reactions should also be tested to confirm the identification further.

If the crystals are found to be bright between crossed nicols they are anisotropic (doubly refractive). Upon rotating the stage it will be found that the crystals become dark four times during a single rotation. The positions of darkness are the extinction positions. The angle formed by the long axis of the crystal and the nearest nicol vibration axis when the crystal is in an extinction position is known as the extinction angle of the crystal. Extinction angles are between 0° and 45°. The vibration axis of each nicol is marked on the metal frame in which the nicol is mounted.

An anisotropic crystal may have one optic axis (uniaxial crystals) or two optic axes (biaxial crystals). Optic axes are explained by Winchell (472) and Chamot and Mason (281). Uniaxial crystals have two characteristic refractive indices and biaxial crystals have three characteristic indices which are listed in refractive index tables.

To determine the refractive indices of anistropic crystals choose crystals showing the highest order of color since these crystals show the refractive indices which are characteristic for the compound. For color charts showing the polarization colors in the different orders see Johannsen (335*a*). Having chosen such a crystal by viewing the crystals through crossed nicols, rotate the stage until the crystal is in an extinction position. Remove the analyzer and by placing a dark paper over part of the mirror determine whether the crystal in this position has a higher or lower refractive index than the mounting liquid. Replace the polarizer or analyzer and rotate the crystal 90° to the other extinction position. Remove the analyzer as before and determine whether the refractive index of the crystal in this position is higher or lower than the mounting liquid. Repeat this procedure with five or six crystals. Mount crystals in liquids of different re-

fractive indices and repeat the procedure until maximum and minimum values are obtained.

If the crystals are uniaxial it will be found that all crystals show the same refractive index when in one of the extinction positions. The refractive index in the other extinction position varies with the position of the crystal. The constant index is designated as ω; the variable refractive index is ϵ. The value which differs most from ω is that chosen for ϵ. If ϵ is greater than ω the crystal is said to be positive; if ϵ is less than ω the crystal is negative.

If no constant refractive index is found the crystals are biaxial. The lowest refractive index found is designated α, and the highest value is γ. A third refractive index β is found by choosing crystals showing color of the lowest order. The refractive index of these crystals in the two extinction positions is determined as before. β is then considered to be midway between the lowest value of γ and the highest value of α. Thus the three refractive indices of a biaxial crystal are determined. Examples of typical results obtained by the above procedures are given in Tables 1 and 2.

TABLE 1

SHOWING PROCEDURE IN IDENTIFYING A COMPOUND (KH_2PO_4) HAVING UNIAXIAL CRYSTALS

Crystal No.	Refractive index of mounting liquid	Refractive index of crystal in first extinction position	Refractive index of crystal in second extinction position
1	1.522	< 1.522	< 1.522
2	1.522	< 1.522	< 1.522
3	1.522	< 1.522	< 1.522
4	1.50	> 1.50	< 1.50
5	1.50	> 1.50	< 1.50
6	1.50	> 1.50	< 1.50
7	1.509	= 1.509	< 1 509
8	1.509	= 1.509	< 1.509
9	1.509	= 1.509	< 1.509
10	1.468	> 1.468	= 1.468
11	1.468	> 1.468	> 1.468
12	1.468	> 1.468	= 1.468

ω = 1.509. ϵ = 1.468.

Other optical properties of crystals may be determined by the methods given by Chamot and Mason (281) and Winchell (472). If crystals in plant sections are to be studied the sections should first be thoroughly washed in distilled water to remove all soluble material. The sections should then be dried between the slide and cover glass to prevent them from curling.

The melting point of crystals may be determined by the use of the micro-melting point apparatus described by Klein (347).

The various tables giving the optical properties of crystals are listed by Chamot and Mason (281) on pages 324, 325, and 327. Among the most complete tables are those of Winchell (472*a*) for inorganic compounds and those in Vols. 1 and 7 of the International Critical Tables for organic and inorganic compounds.

TABLE 2

SHOWING PROCEDURE IN IDENTIFYING A COMPOUND (K_2SO_4) HAVING BIAXIAL CRYSTALS

Crystal No.	Refractive index of mounting liquid	Refractive index of crystal in first extinction position	Refractive index of crystal in second extinction position	Order of color
1	1.522	< 1.522	< 1.522	High
2	1.522	< 1.522	< 1.522	High
3	1.522	< 1.522	< 1.522	High
4	1.488	> 1.488	> 1.488	High
5	1.488	> 1.488	> 1 488	High
6	1.488	> 1.488	> 1.488	High
7	1.494	= 1.494	> 1.494	High
8	1.494	> 1.494	> 1.494	High
9	1.494	= 1.494	> 1.494	High
10	1.497	< 1.497	= 1 497	High
11	1.497	< 1.497	< 1.497	High
12	1.497	< 1.497	= 1.497	High
13	1.495	< 1.495	> 1.495	Low
14	1.495	= 1.495	= 1.495	Low
15	1.495	< 1.495	> 1.495	Low
16	1.496	< 1.496	< 1.496	Low
17	1.496	< 1.496	< 1.496	Low
18	1.496	< 1.496	< 1.496	Low

$\alpha = 1.494.$ $\gamma = 1.497.$ $\beta = 1.495.$

THE IDENTIFICATION OF NON-CRYSTALLINE PLANT CONSTITUENTS

Cellulose.—Cellulose is a relatively inert complex polysaccharide which makes up a large portion of both lignified and unlignified cell walls of the higher plants. It is dissolved and hydrolyzed by concentrations of sulfuric acid above 72 per cent to yield glucose. Pringsheim (423*a*) has given some evidence that certain bacteria digest cellulose through the action of a cellulase which converts cellulose to cellobiose. Cellobiose is then thought to be converted to glucose by a cellobiase. Surprisingly little information is available on this important process.

According to Frey (306), cellulose in the cell wall is probably in the form of submicroscopic elongated anisotropic crystalline micellæ which are usually arranged with their long axis parallel with the cell wall. These micellæ are thought to be imbedded in amorphous isotropic pectic material in the case of parenchyma walls and in isotropic lignin in the case of lignified walls. This conception is based on the observation that the retardation changes with a change in the refractive index of the mounting liquid. (See Chamot and Mason [281] or Winchell [472] for an explanation of retardation.) Anisotropic crystals on the other hand do not show a change in retardation when the refractive index of the mounting liquid is modified. According to Meyer (391) the micellar structure is confirmed by X-ray analysis of cellulose.

It appears that anisotropy in crystals is due to the arrangement of atoms in the crystals whereas that in cellulose-containing walls is due partly to the crystalline structure of the cellulose micellæ and partly to the orderly arrangement of the micellæ.

To determine the orientation of cellulose micellæ in the cell wall mount a section in water, cross the nicols, and insert a first-order red plate with its slow vibration axis at 45° to the vibration axis of the analyzer. Rotate the stage until the cell walls appear brightest. If the cell wall appears blue when the wall is parallel with the slow axis of the first-order red plate the long axes of the micellæ are parallel with the cell wall. This is the arrangement found in most plant walls. Frey (306) found that the micellæ appeared to be irregularly arranged in sieve tubes, and were arranged concentrically around pits.

As will be shown later, the polarizing microscope is very helpful in studying the changes brought about in cell walls by parasitic fungi and bacteria.

Double Refraction Caused by Cellulose.—Cut a free-hand cross-section of a petiole of *Malva parviflora* carrying a sorus of *Puccinia malvacearum.* Mount the section in water and examine through crossed nicols with the slow vibration axis of the first-order red plate at an angle of 45° with the vibration axis of the analyzer. Rotate the stage until the cell walls appear brightest. It will be observed that the fungus has split the cell walls along the line of the middle lamella and has grown in between the cellulose-containing lamellæ, spreading them widely apart. The cellulose-containing lamellæ which are parallel with the slow vibration axis of the first-order red plate appear bright blue; those perpendicular to this axis appear bright orange. This is one method of demonstrating that this fungus is not active in digesting the cellulose of the host.

Similar results will be observed by fixing tissues of sweet potato, which have been rotted by Rhizopus, in absolute alcohol or alcohol-formalin-acetic fixing solution, then running through the usual paraffin process and mounting the sections in water for observation.

The polarizing microscope should also be useful in studying sections of wood which have been invaded by wood-decay fungi. Quantitative analyses indicate that many of these fungi are active in digesting cellulose.

Staining Cellulose with Zinc-Chlor-Iodide.—Mount sections in several drops of zinc-chlor-iodide and add a cover glass. After a few minutes the cellulose-containing lamellæ are stained blue. Lignified or suberized walls are stained yellow by this reagent. The lignin or suberin must be removed before the blue color can be obtained. (See lignin and suberin.) Sections sometimes stain best if left in water for some hours before staining.

To prepare zinc-chlor-iodide dissolve 50 g. zinc chloride and 16 g. potassium iodide in 17 cc. water. Add an excess of iodine and allow to stand several days. The supernatant liquid is poured off the iodine crystals into a brown dropper bottle and is then ready for use. This reagent is said to keep best in the dark. The zinc chloride is thought to convert cellulose to hydrocellulose, the latter being stained blue by the iodine. Certain hemicelluloses are also reported to have been stained by this reagent. Certain of the prepared zinc-chlor-iodide solutions offered by chemical supply houses have been found to be unsatisfactory for staining cellulose.

Staining Cellulose with I-KI and H_2SO_4.—Treat sections in I-KI solution for ½ hour or longer. Mount in I-KI and add a drop of 65 per cent H_2SO_4 at the edge of the cover glass. Walls containing

cellulose stain blue. This reaction is often called the hydrocellulose reaction. It is thought that the H_2SO_4 converts the cellulose to hydrocellulose, which is stained blue by iodine. The cellulose in lignified walls is stained by this method. The method is therefore useful for demonstrating that the cellulose in wood cells occurs in the secondary walls and that the middle lamella of such cells consists mainly of lignin which is stained yellow to orange by this method. Certain hemicelluloses are reported to be stained blue by this reagent. According to Eckerson (296*a*), hemicelluloses are removed by heating sections in 3 per cent H_2SO_4 for 2 hours. The sections should then be washed in water and the hydrocellulose test or zinc-chlor-iodide test applied. Any blue-staining wall material may be considered to be cellulose.

The I-KI solution for the hydrocellulose reaction is made by dissolving 1 g. iodine and 3 g. potassium iodide in 300 cc. of distilled water. The H_2SO_4 solution should contain at least 65 per cent H_2SO_4. Since this acid absorbs water from the atmosphere, concentrated H_2SO_4 often contains less acid than the amount specified on the label. The specific gravity of the concentrated acid should be determined, and the concentration can then be determined by reference to a chemical handbook. Keep the acid solutions tightly stoppered at all times to prevent absorption of water from the atmosphere.

Solution of Cellulose in 72 per cent H_2SO_4.—The cellulose in wood may be dissolved in 72 per cent H_2SO_4, leaving the lignin undissolved. Unfortunately the tissues are usually badly distorted by this treatment, only the lignin in the middle lamella remaining in place. The lignin associated with cellulose in the secondary walls usually floats about as small particles after this treatment.

Solution of Cellulose in Cuprammonia.—Cellulose in unlignified and unsuberized walls may be dissolved by cuprammonia after a treatment of 12 to 36 hours.

Cuprammonia solution is made by saturating a strong solution of ammonia with cupric hydrate. The container should be kept tightly stoppered. We have been unable to produce satisfactory cuprammonia by passing air through ammonia containing copper as is recommended by some workers.

Method of Detecting Cellulose Digestion by Wood-decay Fungi.—Dehydrate the infected sections of wood by placing them in absolute alcohol for about 10 minutes and then drying them on filter paper. After about 5 minutes of drying, place 1 or 2 drops of a solution of

celloidin (dissolved in equal parts of absolute alcohol and ether) on a slide and quickly spread it into a thin layer with a brush. The dried section is then quickly placed on the thin layer of celloidin and is pressed gently with the finger tip to insure uniform contact of the section with colloidin. The slide is then allowed to dry for a day or longer.

The slide is placed in water until the celloidin film with adhering section can be peeled from the slide. The film and adhering sections are then placed in chlorine water for ½ hour. The treatment is followed by washing in water, with subsequent treatment for 15 minutes in 2 per cent sodium sulfite in a hot water bath. The sulfite is removed by washing. Repeat the chlorine-sulfite treatment at least 2 more times until all lignin is removed.

After the above treatment only cellulose should remain in the walls. To insure that no lignin remains, treat one of the sections with I-KI and H_2SO_4 as described above. If all the lignin has been removed the cellulose should stain a bright blue, no yellow-staining lignin remaining. One of the chlorinated sections which has not been stained by I-KI and H_2SO_4 is now stained by some cellulose stain such as Delafield's hematoxylin. The section may then be run up through absolute alcohol and clove oil and mounted in balsam. If cellulose is absent in infected wood or is present in smaller amount in infected wood than in uninfected wood after the above treatment the fungus may be considered to be active in cellulose digestion.

This method is also useful for showing the presence of lignin and the absence of cellulose in the middle lamella of wood cells, the middle lamella being dissolved out by the chlorine-sulfite treatment.

Chlorine water may be purchased from certain chemical supply companies. It should be kept in the dark and should be tightly stoppered. Avoid inhaling chlorine gas more than is necessary.

Non-pectic Hemicelluloses.—The non-pectic hemicelluloses are polysaccharides which are dissolved by 4 per cent NaOH or dilute mineral acids and yield various sugars, or sugars and uronic acids, upon hydrolysis. Unlike pectic substances they do not yield pectic acid on decomposition. The non-pectic hemicelluloses are most abundant in the cell walls of seeds and in lignified tissues. Galactans have been extracted from fungi by Dox (815).

Clayson, Norris, and Schryver (283*a*) found that 4 per cent NaOH, if free from carbonate, caused the solution of at least part of the non-pectic hemicelluloses. The alkali was allowed to act for 2 hours at room temperature. No pectic material was brought into solution

by this treatment. There is a great need for more work on the separation and identification of the non-pectic hemicelluloses.

Perhaps the best method to detect non-pectic hemicelluloses is to treat a large number of sections with 4 per cent NaOH (carbonate free) for about 24 hours at room temperature and then filter off the NaOH solution. Wash the sections in water, neutralize with acetic acid, and stain with 1:1000 aqueous solution of ruthenium red. If any portions of the cell walls are seen to have been removed by the treatment these may be considered to be non-pectic hemicellulose. To insure that no pectic substances have been removed by this treatment, neutralize the NaOH extract with acetic acid and add a solution of $CaCl_2$. If pectic substances are present a precipitate of calcium pectate is formed. There is some doubt as to the reliability of this method for distinguishing non-pectic hemicelluloses and pectic substances, but at the present time it appears to be the most promising method.

According to Thaysen and Bunker (451*a*) and Waksman and Diehm (462*b*) there is good evidence that hemicelluloses may be hydrolyzed by many fungi and bacteria. Some fungi are able to bring about the decomposition of certain hemicelluloses but cannot decompose others. Thus it is probable that different cytases are required to bring about the decomposition of different hemicelluloses.

Pectic Substances.—Pectic substances occur in unlignified cell walls in the middle lamella and in the cellulose-containing lamellæ. They are particularly abundant in certain fruits such as apple and citrus fruits, and in certain fleshy roots such as carrot and turnip.

The nomenclature of the pectic substances is in an exceedingly confused state as is shown in the very complete review by Branfoot (271). Pectose, sometimes called protopectin, is found in the cellulose-containing lamellæ. It is insoluble in water and is hydrolized by acids or by certain fungi and bacteria. The middle lamella pectic material is also insoluble in water but is apparently more resistant to acid hydrolysis than the pectose. Organisms which decompose pectose also decompose the middle lamella pectic material. Whether both substances are decomposed by the same enzyme or whether a different enzyme is required for the decomposition of each substance is a subject which should be investigated since this is one of the first processes which occur when certain parasites invade the host. Further research on the composition of pectose and middle lamella pectic material and on the enzyme or enzymes which decompose these sub-

stances may eventually yield information regarding the cause of the resistance or susceptibility of hosts to parasites.

Upon treatment of pectose with acid a water-soluble substance called pectin results. Pectin is apparently a methoxylated pectic acid. According to Branfoot (271), if pectin or acid-treated middle lamella pectic material is treated with NaOH either one is converted to the soluble sodium salt of pectic acid. Pectic acid yields galacturonic acid, galactose, and arabinose on hydrolysis. The calcium and barium salts of pectic acid and the acid itself form insoluble gels.

The enzyme which causes a solution of the middle lamella pectic material is known as protopectinase. Jones, Harding, and Morse (335*b*), Harter and Weimer (321), and various other workers have shown that this enzyme is produced in abundance by certain pathogenic bacteria and fungi.

Davidson and Willaman (293) found that protopectinase is inactivated at a temperature of 48° C. and that the optimum *p*H for its activity is around 5. Pectinase, the enzyme which converts pectin to its constituent sugars and galacturonic acid, is inactivated at 60° C. and is most active at *p*H 3. This enzyme is also produced by certain bacteria and fungi.

Methods of Detecting Digestion of Pectic Substances by a Pathogene. 1. Ruthenium Red Method.—With a sharp razor blade cut out blocks of host tissue along the advancing margin of invasion so that each block contains about half healthy and half infected tissue. The blocks should be about 1 to 2 cm. square. Fix the blocks in absolute alcohol for about 6 hours, then complete the dehydration by another treatment in absolute alcohol for about 6 to 12 hours. The material is then imbedded in paraffin as in the paraffin method and sections are cut and fixed to a slide. The sections are stained with aqueous ruthenium red 1:5000 until the walls of the healthy tissue are bright red, are washed in water for about 5 minutes, and mounted in Kaiser's gelatin. The edges of the cover glass are then sealed with vas-par or very viscous xylol-balsam. If the pathogene has digested the middle lamella pectic material, the middle lamella will be absent, and if the pectose has been removed the cellulose-containing lamellæ will be unstained. These lamellæ appear bright blue or orange if examined through crossed nicols with the addition of the first-order red plate.

2. Iron Absorption Method.—Pectic substances have a marked tendency to take up ferric salts. Paraffin sections prepared as described above are placed in a 10 per cent solution of $FeCl_3$ for 10

to 20 minutes and are then washed in four or five changes of distilled water during 5 to 6 hours or longer to remove all uncombined ferric salt. Remove the slide from the water and add a drop or two of 2 per cent potassium ferrocyanide to the sections. After about 2 minutes add a drop of 2 per cent HCl to the potassium ferrocyanide. The iron which has been taken up by the pectic substances reacts with the potassium ferrocyanide in the presence of acid to produce a blue precipitate of Prussian blue. In this manner the walls of the uninfected tissues are stained blue while those in which the pectic substances have been digested by the enzymes of the pathogene remain unstained or only lightly stained. The sections are then washed in distilled water for about 10 minutes and are mounted in Kaiser's gelatin and sealed as above.

3. Solution of Cellulose in Cuprammonia.—Place paraffin sections prepared as above in cuprammonia solution for 1 to 3 days until all the cellulose has been removed as shown by examination with polarized light. Wash in water, then in 1 per cent acetic acid for about 5 to 10 minutes to remove the cuprammonia, then in water again. Stain with ruthenium red 1:5000 until the walls of the uninfected tissue stain red. Wash in water and mount in Kaiser's gelatin. The cuprammonia dissolves the cellulose but leaves the pectic substances in place. In infected tissues in which the pectic substances have been digested by the enzymes of the pathogene no wall material remains after this cuprammonia treatment. (The preparation of cuprammonia is described under cellulose.)

The advancing margin of the rot produced in sweet potato by Rhizopus is excellent material for demonstrating the above three methods. Rhizopus produces a solution of the middle lamella and the pectose in the cellulose-containing lamellæ. In cross-sections of petioles of *Malva parviflora* infected by *Puccinia malvacearum* the cellulose-containing lamellæ are split apart by the hyphæ, but we have not been able to determine whether the thin middle lamella is dissolved or is split by pressure. Unlike Rhizopus this fungus does not produce a solution of the pectose in the cellulose-containing lamallæ.

Kaiser's gelatin is prepared by dissolving 5 g. of a good grade of gelatin in 30 cc. of water. Add 35 cc. of glycerin and 0.7 g. of phenol. Warm the mixture for 15 minutes, stirring all the while until all the flakes produced by the phenol have disappeared. Filter while warm through a fine-mesh cheesecloth. The mixture is then stored in a well-stoppered bottle until needed. To prepare a mount, take out a

piece of the gelatin mixture about the size of a grain of wheat and warm it on a slide until it becomes fluid. Place the section in the fluid and add a cover glass. When gelation has occurred, seal the edge of the cover glass with very thick xylol-balsam or melted vas-par.

Lignin.—Very reactive reagents are required to separate lignin from lignocellulose. For this reason the lignin which has been isolated and studied probably does not have the composition and structure of that occurring in the plant. According to Haas and Hill (316), lignin contains hydroxyl, methoxyl, acetyl, and aldehyde or ketone groups, is unsaturated, and contains phenolic groups.

Phillips (414) isolated normal propyl guiacol as a decomposition product of lignin. Treatment of lignin with chlorine produces a lignin chloride which is soluble in 2 per cent sodium sulfite or other alkaline solutions. Lignocellulose is not dissolved by cuprammonia or by the viscose reaction. This suggests that lignocellulose has a different structure from the cellulose in unlignified walls which is readily dissolved by these reagents. Lignocellulose is dissolved by 72 per cent H_2SO_4 or 40 to 42 per cent HCl, the lignin remaining undissolved.

Lignin occurs in the cell walls of wood and bast fibers, and in the walls of vessels contained in succulent tissues. In the walls of wood cells the middle lamella is apparently composed largely of lignin, the secondary walls containing both cellulose and lignin. The location of the hemicelluloses which occur in the walls of wood cells has not been determined.

Aldehyde Reaction.—All studies on the composition of lignin have indicated the presence of an aldehyde group in the lignin molecule. Mount a section in ammoniacal silver nitrate solution. Heat over a steam bath until lignified tissues become dark through the formation of silver. Wash in water, dehydrate in absolute alcohol, and clear with clove oil. Mount in balsam.

The ammoniacal silver nitrate solution is prepared by adding NH_4OH solution to a 10 per cent solution of $AgNO_3$ until all the AgOH precipitate is dissolved. The aldehyde group reduces silver ion to metallic silver.

Maule Reaction.—Treat sections with 1 per cent potassium permanganate solution 10 to 20 minutes. Wash in distilled water 5 minutes. Place sections in HCl (sp. gr. 1.06) for 5 minutes and then wash 10 minutes in distilled water. Mount in a 5 per cent solution of $NaHCO_3$. Lignified tissues stain red. This reaction is apparently specific for lignin. Manganese dioxide is said to be formed in the tissues. The HCl then reacts with the manganese dioxide to produce

chlorine, which reacts with the lignin to form a lignin chloride. In the alkaline bicarbonate solution the lignin chloride is apparently converted to a red compound.

Hydrocellulose Reaction.—This reaction is described under cellulose. If 72 per cent H_2SO_4 is used following I-KI the cellulose stains blue and then dissolves, the lignin remaining undissolved and staining yellow. By the use of this technic it may be demonstrated that much of the lignin in wood cells is in the middle lamella.

The above lignin reactions may be used in studying the action of wood-decay fungi on the cell walls in wood. In general, these fungi are more active in decomposing cellulose than in digesting lignin. Certain species such as *Trametes pini* are very active in decomposing lignin. It has generally been supposed that the fungi producing white rots decompose the lignin of the wood, whereas those producing red rots destroy the cellulose. According to Hawley and Wise (325), this conclusion is not always warranted, the cellulose being largely removed in certain of the white rots.

Chlorination.—Lignin may be removed from the cell walls of wood by alternate treatments with chlorine water and sodium sulfite solution. This technic is described under cellulose. After this treatment the middle lamella is absent, only the cellulose in the secondary layers remaining.

Detection of Lignin Digestion by Wood-decay Fungi.—Place a drop of warm 1½ per cent gelatin sol on a warm slide and quickly spread it to a thin film by the use of a camel's-hair brush. A wet section of decayed wood is quickly placed on the gelatin film and the slide is placed in a closed vessel containing formaldehyde gas for 2 days. (The formaldehyde gas is produced by putting the slide in a Coplin staining jar and then pouring in formalin until it is about ½ inch deep.) The slides are then dried for 2 or more days at room temperature. After the gelatin is dry, add several drops of $ZnCl_2$ in twice its weight of concentrated HCl and heat over steam for several minutes. Do not agitate the slide and do not heat sufficiently to produce boiling since such treatments tend to disrupt the sections. The slides are then gently washed in water and are examined through crossed nicols. If the walls remain anisotropic the cellulose has not been entirely removed and the zinc chloride treatment must be repeated until the walls become isotropic. Stain one of the treated sections with I-KI and H_2SO_4 as a further check on cellulose removal. If no blue staining of the walls results, the cellulose removal may be considered complete. Stain one of the sections (which has not had

the I-KI and H_2SO_4 treatment) with a 1:1000 aqueous solution of ruthenium red, wash in water, and mount in Kaiser's gelatin. In uninfected tissues this treatment removes the cellulose from the secondary walls and leaves the red-stained lignin of the middle lamella on the slide. Absence or diminution of middle lamella material in infected tissues after the above treatment may be considered as evidence that the fungus has modified the lignin of the middle lamella. No method has been found for detecting lignin digestion in the secondary walls since the lignin in the secondary walls does not remain in place on the slide after the above treatment.

Suberized Cell Walls.—Suberized cell walls contain substances which take up fat-soluble dyes such as sudan IV. According to Rhodes (425), suberized potato walls contain very little neutral fat, most of the chloroform-soluble material being made up of fatty acids. After extraction with chloroform the walls showed little tendency to stain with fat-soluble stains. Upon boiling with alcoholic KOH the suberin is decomposed and salts of several complex acids are formed. This is believed to indicate that suberin is a complex substance made up mainly of complex acids. After the constituents of suberin have been removed by KOH the remainder of the wall is considered to be cellulose since it is soluble in cuprammonia. Boiling suberized walls in concentrated KOH followed by treatment with acid is said to produce crystals of phellonic acid. This method may be used to distinguish between suberin and cutin, no phellonic acid crystals being formed from cutin.

Suberization occurs in the walls of cork cells, outer cells of roots, endodermal cells, and wound cork. A layer of wound cork is often formed in host tissues, adjacent to tissues infected by fungi or bacteria. Such wound cork may inhibit further invasion of the host by a pathogene.

Method for Demonstrating Wound Cork.—Cut sections of infected tissues and mount in sudan IV staining solution. Seal the cover glass with vas-par or very thick xylol-balsam. After a few hours the suberized walls of the wound cork are stained red. Several days may be required for maximum staining. Cross-sections of brown rot cankers, or diamond canker on French prune are excellent for showing the formation of wound cork.

To prepare the sudan IV staining solution make a saturated solution of sudan IV in 95 per cent alcohol and then add an equal volume of glycerin. Filter or decant and use the clear solution for staining. If the sudan IV crystallizes out, the staining solution may be diluted

with a solution containing equal parts of 95 per cent alcohol and glycerin.

Cutin.—Like suberin, cutin takes up fat-soluble stains such as sudan IV. Cutin is apparently a mixture of numerous compounds. Fatty acids, alcohols of high molecular weight, and hydrocarbons are among the compounds which have been most commonly isolated from the cuticle. The cuticle reacts with Schiff's reagent, indicating the presence of aldehydes.

Cutin occurs in the cuticle of higher plants. All the available evidence indicates that pathogenes are unable to digest the constituents of cutin. Most workers who have studied the penetration of the cuticle by pathogenes consider that germ tubes force their way through the cuticle rather than by solution of the cutin constituents.

Staining with Sudan IV.—The cuticle may be stained with sudan IV as described under suberized cell walls. This method is useful in studying the penetration of the cuticle by germ tubes.

Reaction with Schiff's Reagent.—Place a section in a drop of Schiff's reagent on a slide. The cuticle and other structures containing aldehydes are stained red.

To prepare Schiff's reagent make a 2 per cent solution of rosaniline hydrochloride or basic fuchsin in water. This solution is exposed to SO_2 gas until the solution becomes yellow. After filtering, the reagent is ready for use.

Reaction with Ammoniacal $AgNO_3$.—The cuticle also reacts with this reagent for aldehydes. The preparation and use of ammoniacal $AgNO_3$ is described under lignin.

Chitin.—Chitin is an inert substance found in animals and in the walls of most fungi. Upon heating in a saturated solution of KOH at a temperature around 180° C., chitin is thought to be converted to a substance called chitosan which has characteristic staining properties with iodine and other reagents. Upon being heated in concentrated HCl chitosan yields glucosamine hydrochloride and acetic acid.

According to Von Wisselingh (476), chitin occurs in the walls of most fungi. It was not found in the Oomycetes studied.

Chitosan Reaction.—Cut sections of host tissues containing fungus mycelium. Place these sections in a saturated aqueous solution of KOH and heat in an autoclave at 15 lb. steam pressure for 3 hours. (One hour has been found sufficient for some fungi.) The treatment is said to convert chitin to chitosan. Then place the sections in 95 per cent alcohol for ½ hour or longer to harden them. Wash in water

about 5 minutes to remove the alcohol and soak sections in I-KI solution for about 15 minutes. Mount the sections on a slide in I-KI solution and draw 5 per cent H_2SO_4 under the cover glass. Mycelium containing chitosan is stained purple. (See cellulose for method of preparing I-KI solution.) Other methods of staining chitosan are described by Von Wissellingh (476).

Brown rot cankers and sori of *Puccinia malvacearum* have been found to be satisfactory material for demonstrating this method. It has also been used satisfactorily on wood-decay fungi, the mycelium standing out very distinctly.

Chitosan Nitrate.—Convert chitin to chitosan by the use of KOH as in the preceding method. Place sections in 95 per cent alcohol 12 hours or longer to harden and to remove alkali. Wash in water 5 minutes. Mount a section in a drop of 50 per cent HNO_3. Add a cover glass and carefully heat the slide over steam for 5 minutes. Remove the flame from beneath the beaker supplying the steam and allow the slide to cool very slowly over the hot water for about ½ hour. The mycelium will be found to have dissolved and in its place will be found crystal masses which are said to be chitosan nitrate. The smaller of the crystal masses are apparently made up of radially arranged minute crystals, since each mass shows a dark cross when observed through crossed nicols The sorus of *Puccinia malvacearum* is good material for demonstrating this method. It is probable that any fruiting body containing chitin would yield a conspicuous mass of these crystals.

Callose.—Little is known as to the composition of callose. Most workers have detected it only by its avidity for certain dyes. Thomas (453) has reviewed the literature on callose. It is reported to be insoluble in water, alcohol, alkali carbonates, and cuprammonia, and to be soluble in weak, cold solutions of NaOH and KOH, concentrated solutions of $CaCl_2$, $SnCl_2$, and H_2SO_4. In ammonia it is reported to swell to form a gelatinous substance. Apparently in certain tissues callose is insoluble in alcoholic potash. In certain cases callose-containing tissues must be treated with alcoholic potash or oxidizing agents, or both, before the characteristic staining of callose can be obtained. Thomas reported that he was able to obtain callose-staining reactions in Sclerotinia mycelium after the following treatment: Boil in 95 per cent alcohol, then in ether, and then in 1 per cent alcoholic potash. After neutralizing and washing, callose is stained by resorcin blue (lacmoid).

Callose is said to yield glucose on hydrolysis. It is possible that the dextrans which various workers (462*b*) have reported in fungi may really be callose.

A substance which is stained by resorcin blue occurs in sieve plates, as "plugs" in pollen tubes and various species of the Peronosporeae, and in the walls of certain root hairs. Such material has always been considered to be callose. It must be admitted, however, that the evidence as to the nature of callose and as to whether all substances which stain with resorcin blue are callose is very incomplete. The composition of various fungi has not been studied sufficiently to draw any general conclusions as to the presence of callose in fungus cells. Thomas gave rather good evidence that it is present in Sclerotinia but he did not detect it in Fusaria.

Staining Callose with Lacmoid.—The staining solution is a 1:1000 solution of lacmoid (resorcin blue) in 50 per cent alcohol. Cut sections of lettuce infected with *Bremia lactucae*. Place the sections in the staining solution for several days. Rinse sections in water and mount in Kaiser's gelatin. Seal the edge of the cover glass with thick xylol-balsam or vas-par. The mycelium will be found to contain blue-stained "plugs" of material which is considered to be callose. If callose is to be detected in the walls of hyphæ the methods used by Thomas (453) should be tried.

Starch.—According to the review given by Haas and Hill (316), starch consists of 66 per cent amylose and 33 per cent amylopectin. Amylose is said to be soluble in water and gives a bright blue color with iodine. It is converted completely into maltose by barley diastase at 50° C. Amylopectin, when made into a paste with hot water, gives a bluish-black precipitate with iodine. It is changed to phosphoric acid and alpha-beta-hexa-amylose by barley diastase.

Buchanan and Fulmer (496) state that amylase (diastase) is considered to be a mixture of two distinct enzymes which can be separated by ultrafiltration. The amyloclastic or amylolytic enzyme transforms starch to dextrins, and the saccharifying enzyme transforms dextrins to maltose. Maltose is hydrolyzed to glucose by maltase. Both diastase and maltase are produced by many fungi and bacteria.

Starch grains are anisotropic and are apparently made up of minute crystals or micellæ arranged radially. Those crystals in the positions of extinction appear dark and produce a dark cross in the

starch grain when viewed through crossed nicols. If the first-order red plate is used the starch grain takes on a striking appearance, two sectors of the grain being a brilliant blue and two a bright orange. The micellæ or minute crystals in the blue sectors have their slow vibration axis approximately parallel to the slow vibration axis of the first-order red plate, whereas the fast vibration axis of these micellæ is perpendicular to the slow vibration axis of the first-order red plate. The micellæ in the yellow sectors have their slow vibration axis approximately perpendicular to the slow vibration axis of the first-order red plate and their fast vibration axis parallel to the slow vibration axis of the first-order red plate. Polarization colors are more fully explained by Chamot and Mason (281) and Winchell (472).

Demonstration of Starch Digestion by Pathogenes.—Many pathogenes are able to produce enzymes which catalyze the hydrolysis of starch. To demonstrate starch hydrolysis by a pathogene, cut sections of tissue so that part of the section contains uninfected starch-containing tissues and the rest of the section contains infected tissues. Mount the section in I-KI solution. Starch stains blue to black. If the pathogene is active in starch hydrolysis there will be much less starch in the infected tissues than in the uninfected portions of the sections.

Longitudinal-radial sections through the sorus of *Puccinia malvacearum* on the petiole of *Malva parviflora* are excellent material for showing starch digestion by a rust.

To prepare I-KI solution, dissolve 2 g. of KI in 100 cc. water and then dissolve 0.2 g. of iodine in this solution.

Glycogen.—Glycogen is thought to be made up of polymerized maltose. Glycogenase, the enzyme catalyzing the hydrolysis of glycogen, may be identical with diastase. When hydrolyzed by acids glycogen yields glucose.

Glycogen is quite similar to starch. It is widely distributed in animals and is present in bacteria and fungi where it apparently serves as reserve food. In yeast it is sometimes said to make up as much as 30 per cent of the dry weight. Haas and Hill (316) report that in spores of Mucor and in certain sclerotia glycogen does not appear until growth has commenced. This is considered as evidence that it is an intermediate stage in a reaction rather than primarily a storage material.

Glycogen is said to be soluble in hot water and insoluble in

alcohol. It is stained red to brown by I-KI and does not reduce Fehling's solution.

Staining Glycogen with I-KI.—Mount sections containing fungi or bacteria in I-KI or take fungi or bacteria from cultures and mount in I-KI solution. The glycogen appears as red to brown staining granules in the cytoplasm. Glycogen is abundant in the asci of Ascomycetes, *Pseudopeziza medicaginis* in alfalfa being good material for demonstrating the distribution in asci.

The addition of 1 drop of 10 per cent NaCl to 2 drops of the I-KI solution is reported to cause brighter staining of glycogen.

Gums.—According to Norman (403*a*), gum arabic contains about 13 per cent uronic acids, 26 per cent arabinose, and 60 per cent galactose. From gum tragacanth he obtained (403*c*) arabinose and uronic acids. Haas and Hill (316) report cherry gum to yield chiefly arabinose with some xylose also present. They point out that the wood of the cherry yields chiefly xylose on extraction with alkali and that the origin of the gum is therefore difficult to explain. In gummosis of both Prunus and Citrus species conspicuous gum pockets occur in the outer wood. All the cell structures in these pockets appear to have been dissolved. We have found little evidence to indicate whether the gum is derived from the cellulose, lignin, or hemicelluloses in the wood, or whether it is made up of the decomposition products of all these substances, as histological work suggests. The composition of gum is similar to that of certain hemicelluloses, each containing arabinose, galactose, and uronic acids. The pectic substances which were supposedly in the walls of the cambium cells before they were differentiated to form wood cells also contain these constituents. There is apparently an excellent opportunity for biochemical studies on the conversion of cell constituents into gum and the enzymes or other agencies which bring about this decomposition. According to Eckerson (296*a*), cherry gum contains a cytase which can cause the decomposition of the hemicellulose in the walls of certain seeds.

The gums dissolve or are dispersed in water to form a sol or gel. They are insoluble in alcohol.

Pentose Reaction.—Since gums yield pentoses on acid hydrolysis a pentose reaction may be used as a method of locating gum in diseased tissues. HCl converts pentosans to pentose and then converts the pentose to furfuraldehyde which reacts with various phenols to give colored condensation products.

By means of the sliding microtome cut cross-sections of gumming twigs. Keep the knife and the surface of the twig flooded with 95 per cent alcohol to precipitate the gums in the tissues. Transfer the sections to a vial containing 95 per cent alcohol. Place a section on a slide and allow it to become dry or nearly dry. Add 1 or 2 drops of 4 per cent orcinol solution and then draw off most of the orcinol solution from under the cover glass by means of filter paper. Add a drop of concentrated HCl (sp. gr. 1.18). Remove the excess acid from the edge of the cover glass and seal with vas-par. The gum and the pentosans in lignified tissues are stained blue. The middle lamella of lignified walls stains more heavily than the secondary walls, suggesting that much of the pentosan in lignified tissues is in the middle lamella. The reason for the absence of stain in the parenchyma walls containing pectic substances is not clear. Pectic substances are hydrolyzed by HCl, yielding among other compounds the pentose arabinose. Whether the lack of staining of parenchyma is due to the low concentration of pentose or to a relatively slow rate of hydrolysis of pectic substances has not been determined. It is possible that the color reaction with orcinol may really be due to aldehydes associated with lignin rather than to pentoses. (See "Pentoses and Pentosans."

Wound Gum.—A substance called wound gum is often found in the vessels of plants adjacent to wounds, and in wood being invaded by wood-decay fungi. Apple wood infected with *Polystictus versicolor* contains an abundance of this material.

It is insoluble in water and stains red with phloroglucinol and HCl.

Demonstration of Wound Gum.—By the use of a sliding microtome cut longitudinal sections of the advancing margin of Polystictus decay in a limb of the apple tree. Mount a section in a saturated solution of phloroglucinol in 18 per cent HCl. Seal with vas-par. If the wound gum is not stained red after a few minutes, heat another section in a little of the phloroglucinol-HCl mixture to hasten the reaction and after cooling seal with vas-par. (Do not allow HCl fumes to escape in a room containing microscopes as they corrode the metal surfaces very rapidly.)

Tannins.—Upon acid hydrolysis, tannins usually yield glucose and various other compounds. Most tannins are oxidized rather readily to yield colored compounds. Tannins reduce Fehling's solution and other easily reduced reagents and form dark green, blue, or black inks with ferric salts. In hot water they form colloidal sols. Other reactions of tannins are given by Haas and Hill (316).

Tannins are especially abundant in the bark of certain trees and in galls, and also they often accumulate near wounds and infected tissues. Some tannins are quite toxic to certain fungi, and several workers have suggested that they may have a part in the resistance of a host to certain pathogenes.

Reaction with Ferric Salts.—Mount a section of infected tissue in water. Tannins frequently appear yellow to brown in color. Draw a drop of 10 per cent ferric chloride under the cover glass by the use of filter paper. The tannins are stained dark green, blue, or black. The advancing margin of wood decay in fig wood is suitable material for showing the accumulation of tannins in the host tissues adjacent to infected tissues.

Sugars. *Alpha-naphthol Test for Carbohydrates.*—Place material to be tested on a slide; add a drop of 4 per cent alcoholic solution of alpha-naphthol and a drop of concentrated H_2SO_4; add a cover glass and heat over steam until a purple to blue color begins to appear in the material. The color rapidly diffuses out of the carbohydrate material, and the test is therefore not of much use in determining the exact location of carbohydrates.

According to Haas and Hill (316), the reaction depends on the production of furfural by the action of H_2SO_4 on the carbohydrates. The furfural then reacts with the alpha-naphthol to produce the colored compound. The reaction is said to be given by all true carbohydrates and substances which contain a carbohydrate complex, such as glucosides and certain proteins.

Tests for Pentoses and Pentosans.—1. The orcinol test described earlier under "Gums" may be used for detecting pentoses and pentosans. The reaction may be hastened by heating the slide with steam.

2. Mount a section in a saturated solution of phloroglucinol in 18 per cent HCl. Add a cover glass and heat over steam. Pentoses and pentosans react with the phloroglucinol to produce a red compound.

The reliability of the above methods for detecting pentoses and pentosans has not been demonstrated. Various workers have indicated that aromatic aldehydes which produce a red color in the presence of phloroglucinol and HCl occur in lignified walls. The question thus arises as to whether the color occurring when orcinol or phloroglucinol is added to lignified walls is due to the aldehydes associated with lignin or to pentoses.

Seliwanoff's Reaction for Detecting Ketoses.—Place a drop of saturated aqueous solution of resorcin and a drop of concentrated HCl

on a slide. Add a section from a ripe apple and cover with cover glass. Heat over steam 10 minutes until a red color results in the solution. The red color diffuses out of the section very rapidly, and therefore the test cannot be used for determining the exact location of the ketose.

According to Haas and Hill (316), this reaction is given by all keto-hexoses and by carbohydrates such as sucrose and raffinose which give rise to keto-hexoses on hydrolysis.

Identification of Specific Sugars.—Sugars react with phenylhydrazine to form crystalline osazones. The osazones of the different sugars can be identified by their melting points, refractive indices, and other crystal characters.

Mix a drop of phenylhydrazine hydrochloride solution and a drop of sodium acetate solution on a slide. Mount a section of ripe apple fruit in the liquid and heat over steam for 1 hour. Allow the slide to cool. Crystals of osazone will be formed in the cells and in the solution surrounding the section. The section and surrounding crystals may be washed through several changes of distilled water during a period of several days to remove water-soluble material. The section and crystals should then be dried and the crystals identified by the use of the methods described under "Identification of Crystals."

The phenylhydrazine hydrochloride solution is prepared by dissolving 1 g. of phenylhydrazine hydrochloride in 10 cc. of glycerin by heating in a water bath until solution results. The solution is then filtered.

The 10 per cent sodium acetate solution is prepared in the same way, using glycerin as a solvent. Both solutions should be stored in the dark.

The melting points of the osazones and other specific reactions for sugars are given in Table 3.

Glucose, fructose, and sucrose are the sugars which are commonly found free in plants. The other sugars usually occur in polysaccharides which must be hydrolyzed before the sugars can be detected.

Proteins.—Most of the microchemical reactions for proteins are due to the amino acids making up the proteins. Thus not all the reactions are given by all the proteins because of the fact that proteins differ in the amino acids which they contain.

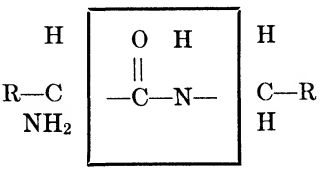
Biuret Reaction.—Place a drop of 10 per cent $CuSO_4$ on a slide and add some young sporangia of Rhizopus. After 10 minutes rinse off the $CuSO_4$ and mount in a drop of 2 per cent NaOH. A blue-violet color results in the dense protoplasm.

TABLE 3

METHODS FOR IDENTIFYING SPECIFIC SUGARS

Sugar	Method
Arabinose	Reacts with diphenylhydrazine to produce a diphenylhydrazone which melts at 204–205° C.
Xylose	1. Osazone melts at 161° C. 2. Oxidize with bromine and boil with cadmium carbonate to produce crystals of a sparingly soluble double salt.
Rhamnose	Osazone melts at 180° C.
Glucose	Yields same osazone as fructose and mannose. This osazone melts and decomposes at 204–205° C. Some of the clusters of crystals resemble wheat sheaves in appearance. According to Klein (347), glucose and fructose yield an osazone after heating 10 minutes. Sucrose is hydrolyzed and yields the same osazone after heating 1½ hours.
Fructose	Klein (347) uses 10 per cent methylphenylhydrazine hydrochloride in glycerin and 10 per cent sodium acetate as in use of phenylhydrazine hydrochloride. Heat over water bath 10 minutes. An osazone is formed which has yellow to brownish red crystals. The crystals may be single flat needles, or in sheaves or sphaerites. The melting point is 158–160° C. This reaction serves to distinguish fructose from glucose and mannose.
Galactose	1. Yields a methylphenylhydrazone melting at 190-191° C. 2. Yields mucic acid crystals on oxidation by concentrated HNO_3. Mucic acid crystals decompose at 206° C. (See Haas and Hill [316] for technic.)
Mannose	An insoluble phenylhydrazone is quickly formed upon treatment with phenylhydrazine hydrochloride and sodium acetate. These crystals are fine prisms which melt at 195-200° C. An excess of phenylhydrazine converts the hydrazone to glucosazone.
Sucrose	Does not react with phenylhydrazine until hydrolyzed. Does not show reducing action. See glucose for method of detecting sucrose.
Maltose	Osazone crystals are broad needles and melt at 206° C.
Isomaltose	Osazone melts at 150° C.
Cellobiose	Osazone melts at 198° C.

The reaction is apparently due to the peptide linkage



and is given by all native proteins. As the protein chains become shorter the color changes toward pink. The color is usually not detectable in the cell unless the proteins are in rather high concentration as in dense protoplasm.

Millon's Reagent.—Proteins or amino acids containing tyrosine are stained red by this reagent. Since other phenolic compounds are stained red by this reagent it cannot be considered as specific for proteins.

Cut thick sections of the chlorotic portion of tobacco leaf infected with mosaic. Place section in Millon's reagent, add cover glass, and warm gently. The nucleus and cell inclusions in the hairs are stained red.

To prepare Millon's reagent dissolve 1 cc. of mercury in 8 cc. of HNO_3 under a hood. Dilute with an equal volume of water. According to Eckerson (296*a*), the reagent does not keep well but may be made effective again by adding a few drops of potassium nitrite or sodium nitrite solution.

Xanthoproteic Reaction.—Mount young Rhizopus sporangia in concentrated HNO_3. Proteins containing a readily nitrated benzene ring are nitrated to form yellow compounds. If ammonia is now drawn under the cover glass the color is intensified to orange. The reaction is given by proteins containing tryptophane or tyrosine or by these amino acids. According to Klein (347), the reaction is also given by various other compounds such as resins and alkaloids.

Vanillin Reaction.—Place a cross-section of rust-infected Malva petiole in a drop of 1 per cent vanillin for 5 minutes. Remove the excess vanillin with filter paper and add a drop of half-diluted H_2SO_4 and a drop of 10 per cent $Fe_2(SO_4)_3$ solution. Add a cover glass and heat gently. After a few minutes, substances which contain the indol ring are colored red, violet, or blue.

Vanillin is an aromatic aldehyde which reacts with an indol-containing substance to produce the color. The indol ring is contained by tryptophane, and the only proteins giving this reaction are those

which contain tryptophane. The dense protoplasm of the rust is stained by this treatment.

Feulgen's Reaction for Thymonucleic Acid.—This method has been used by numerous workers to stain chromatin which contains thymonucleic acid. It was used by Smith (447) to stain the nucleus of virus-infected tissues in order to distinguish the nucleus from the *X*-bodies. The reaction is discussed by Westbrook (468). He recommends the use of fixing solutions which do not contain strong oxidizing agents. Sublimate-acetic solution or fixing solutions containing formalin are said to be satisfactory if tissues are washed thoroughly after fixation. Microtome sections fixed to the slide with egg albumin are hydrolyzed in normal HCl at 60° C. for 4 to 15 minutes. This treatment is said to split up the thymonucleic acid of chromatin into purine bases and thymic acid. The latter is soluble in water but leaves insoluble remains with free aldehyde groups. After washing in water the sections are treated with Schiff's reagent (described under "Cutin"). The aldehydes react with Schiff's reagent to produce a purple compound. Yeast cells which do not contain thymonucleic acid but contain a pentose-nucleic acid are said to be unstained. Bacteria are also unstained. The reaction is said to be useful for distinguishing the minute nuclei in protozoa. Light green is recommended as a counterstain.

Fats.—Fat globules in the host or pathogene may be detected by placing infected sections in the sudan IV staining solution described under "Suberized Cell Walls." The fat globules and other fat-like substances are stained red by this stain. Parat (409) describes various methods for differentiating various fats and fat-like substances. Other papers on this subject are listed in the Bibliography.

Fat soluble dyes have also been used by Hill (332), Knight (354), and others for detecting spray oils in tissues.

Preparing Solutions of Acids, Alkali, Alcohol, Etc.—Determine the specific gravity of the concentrated solution found in the laboratory, maintaining the temperature at that given in the specific gravity tables found in chemistry handbooks.

The specific gravity having been determined, the concentrations of the solution can be determined by reference to the table. If desired, the temperature of the solution may be determined and then a correction made for the difference in temperature as described in chemistry handbooks. The concentration of the solution being known, the following method may be used in making up more dilute solutions:

Take the number of cubic centimeters of solution equal to the per-

centage desired. Add enough distilled water to make a volume equal to the percentage concentration of the concentrated solution.

For example, to make a 50 per cent solution of H_2SO_4 from a 72 per cent solution take 50 cc. of the 72 per cent solution and add enough water to bring the volume up to 72 cc.

MICROMETRY.

Microscopic objects are usually measured by the use of the ocular micrometer, the filar micrometer, or the camera lucida apparatus. Preparatory to the use of any of these instruments it is necessary to carry out the operation of calibration. Since the main error in calibration is usually the worker's judgment as to the limits of the length units, and since this error is likely to be the same whether the length being measured is great or small, it is evident that the percentage of error will be much greater if the length measured is small. Consequently, in calibration work, it is desirable that the length measured be as long as possible and that the value of the smaller divisions on the micrometer be computed instead of measured. For example, let us suppose that one is liable to make an error of 0.001 mm. at each end of the length being measured, which may be an error of 0.002 mm. in the measurement. If one is measuring a length of 1 mm. this error may be 0.2% but if measuring a length of 0.5 mm. the error may be 0.4%.

To calibrate the ocular micrometer, fasten the stage micrometer on the stage of the microscope and place the ocular micrometer in the ocular. Move the stage micrometer until one extremity of the stage micrometer is directly under one extremity of the ocular micrometer. Measure as great a length on the stage micrometer as possible and compute the length measured by the smaller divisions on the ocular micrometer. For example, if 50 of the smallest divisions on the ocular micrometer just cover 66 of the 0.01 mm.-divisions on the stage micrometer, then one of the smallest divisions on the ocular micrometer measures a length of $(66\times0.01)\div50=0.0132$ mm.$=13.2$ microns. Thus an object under the microscope which has a length that is exactly covered by three of the smallest divisions on the ocular micrometer is 39.6 microns long.

If greater accuracy is desired a higher-powered objective or ocular must be used. The calibration is of course useful only for the objective-ocular combination through which it was made, and a separate calibration must be made for each combination. It is also necessary

to recalibrate the ocular micrometer when the tube length of the microscope is changed, a given calibration being applicable only for the tube length at which it was made.

The filar micrometer is calibrated in a similar manner, the micrometer replacing the ocular in the microscope. The rotating drum on the filar micrometer is set at zero, and the zero graduation in the ocular is placed directly over the zero graduation on the stage micrometer. The drum is then slowly rotated until the zero graduation on the filar micrometer has passed over nearly as many of the graduations on the stage micrometer as the range of the filar micrometer will permit. The range of the filar micrometer should have been determined by preliminary tests. When the zero graduation on the filar micrometer is directly over the graduation on the stage micrometer, note the reading on the drum and add this to (the number of turns on the drum times 100). This sum represents the number of the smaller divisions on the drum which have been passed during the measurement. The distance on the stage micrometer which the zero graduation on the filar micrometer has passed is then recorded and the distance measured by the smallest division on the drum is computed. For example, if 50 of the 0.01-mm. graduations on the stage micrometer are passed over by the zero graduation when the drum is rotated 15 times plus 10 of the small divisions on the drum, then $50 \times 0.01 = 0.5$ mm. are passed over when $(15 \times 100) + 10 = 1510$ of the small divisions on the drum are passed. Then one division on the drum measures a distance of $0.5 \div 1510 = 0.000331$ mm. $= 0.331$ micron.

When the camera lucida is used for measuring microscopic objects the instrument is first calibrated by reflecting an image of the stage micrometer on a paper placed on the table in the same position used in drawing. The angle of the mirror and the length of the bar should be recorded and the same positions should be used throughout. The position of the most distant graduations on the paper should be marked with a sharp pencil, and the distance between these marks on the paper should be measured with a millimeter rule. The magnification of the drawing and the length in the microscopic object represented by a millimeter on the paper is easily computed from these data. For example, if 0.05 mm. on the stage micrometer is magnified to give an image on the paper which is 50 mm. long, the magnification is $50 \div 0.05 = 1000$, and 1 mm. on the paper represents a length of $0.05 \div 50 = 0.001$ mm. $= 1$ micron in the object to be measured.

MEASURING SPORES

When suspended in water, spores tend to move about and are therefore very difficult to measure accurately. This difficulty may be obviated by making a suspension of the spores in clear water agar which has been melted and then cooled to 45° C. A drop of the melted agar containing the spores is placed on a slide which has been heated to about 60° C. The edge of a second slide is then quickly drawn across the drop of agar to spread it as a thin film on the first slide. As soon as the agar film solidifies, a drop of water and a cover glass are added and the spores are ready for measurement.

Sherbakoff (223) has recommended the following method for holding spores in a single plane: The concave cavity in a warm, hollow, ground slide is filled with melted 3% water agar and a cover glass is added. When the agar has solidified, the cover glass is removed. A small drop of a water suspension of spores is then spread on a clean cover glass and left until the water has almost completely dried and the surface has assumed a dull appearance. The cover glass is then quickly inverted over the agar and the spores are held stationary in a single plane. Such a preparation is very satisfactory when one desires to photograph or measure spores. Colley (46) recommends the use of a microprojector for measuring spores. The magnified image of the spores is thrown on a screen and the measurements are made on the screen.

THE DARKFIELD MICROSCOPE

When a beam of light from the sun falls upon dust particles in the atmosphere of a darkened room, the light is diffracted by the particles, thus producing a bright halo around them and demonstrating the presence of minute particles which would not be observed in ordinary light. The darkfield condenser similarly throws a beam of bright light at an angle of about 45° on the organisms mounted on the microscope slide, and the halo produced around even very minute organisms is quite conspicuous. The fact that the beam of light from the condenser does not pass directly up through the objective causes the organisms studied to appear very bright against a dark background. The motility of organisms and the Brownian movement of organisms and colloidal particles are strikingly demonstrated by the darkfield microscope.

Microscopic slides must have the thickness prescribed by the maker of the condenser in order that the light from the condenser

may be most intense in the liquid on the surface of the slide. Immersion oil should be used between the slide and the condenser in order to reduce refraction.

When using an objective having a short working distance, it is necessary to place a funnel stop or iris diaphragm in the objective to prevent direct light from the condenser from passing through the objective.

A lamp furnishing light of high intensity is desirable for darkfield work.

PHOTOMICROGRAPHY

In general, structures which can be clearly seen through a microscope can be clearly reproduced in a photograph. As the magnification increases, the difficulty of producing sharp photographs increases. Consequently, photographs taken through the higher-powered objectives are often not as clear as desired.

The first requisite for successful photomicrography is a good microscope. If one is unable to clearly see the structure of an organism through the microscope, it is quite useless to try to photograph it by the use of visible light. Good results are obtained with apochromatic objectives and periplan or compensating oculars. Homal oculars are reported to give the best results in photographic work. A well-corrected condenser is also necessary when using the higher-powered objectives. When using the oil immersion objective, immersion oil should be placed between the condenser and the slide as well as between the objective and the slide.

We prefer a Welsbach gas mantle as a source of light. If the microscope is in a horizontal position the microscope mirror is removed and the lamp is placed in front of the condenser, thus allowing the light to pass directly from the lamp into the condenser. If the organism to be photographed is mounted in water or other liquid media, it may be necessary to keep the slide in a horizontal position in order that the cover glass may not slide off. In such a case the microscope must be vertical and the mirror must be used to reflect the light into the microscope.

A cloth should usually be wrapped about the connection between the camera and the microscope in order to prevent the entrance of light from the room. When possible the photographic apparatus should be in a darkened room, as focusing is easier in the dark.

If considerable depth of focus is desired, a low-powered objective should be used and the desired magnification should be obtained by

changing the ocular or adjusting the bellows of the camera. If sharp definition and high magnification of a single plane are desired, high-powered oil immersion objectives and oculars of sufficiently low magnification to produce a clear image should be used. In any case, it is undesirable to use an ocular of such high magnification that a hazy image is produced. If the highest-powered objective and ocular giving a clear image does not give sufficient magnification, the bellows of the camera should be drawn out until the desired magnification is obtained.

The position of the condenser and diaphragm is regulated to give the clearest image just as in visual work with the microscope.

A dark-colored cloth is placed over the ground glass of the camera and the head of the worker, and the image is focused on the ground glass by turning the focusing screws on the microscope. For best results the clear central portion of the ground glass should then be examined by means of a hand lens and the focusing screws turned until the image appears sharp when viewed through the hand lens.

The contrast between the object to be photographed and its background may often be increased by the use of colored light filters. For example, hematoxylin has been found to transmit violet, blue, orange, and red, but to absorb green. If a green filter is used which transmits only the wave lengths in the green, all the light passing through the stained portions of an organism is absorbed by the filter while the green light passing through the unstained portion passes through the filter and acts on the photographic plate. Thus the contrast between the stained and unstained portions of an organism is increased. Probably, for those who do not have spectroscopic equipment, the most practical way to determine the proper filter is to successively place each of the different colored filters between the light and the microscope and compare the contrast produced by each in the preparation to be photographed. A combination of several of the filters may sometimes produce best results. Dr. M. W. Gardner finds that a green Wratten "B" filter gives good results when photographing unstained preparations.

Ordinary plates are sensitive to violet and blue; orthochromatic plates are sensitive to greenish yellow in addition; and panchromatic plates are more or less evenly sensitive to all colors. Thus ordinary plates are suitable for use with violet or blue filters; orthochromatic plates for use with green or greenish yellow filters; and panchromatic plates are desirable for use with orange or red filters and may be used with any filter.

The time of exposure must be determined by trial. Usually 10 or more seconds are required for low-power work and 1 to 2 minutes may be required for higher magnifications.

Every effort must be made to prevent vibration of the apparatus during exposure of the plate. A black card should be placed between the light and the microscope to regulate the entrance of light. The card is inserted before the plate is uncovered. After the plate is uncovered and vibration of the apparatus has ceased, the card is removed and the exposure made. The card is then replaced to stop the exposure. . By this means all vibration is prevented.

DRAWING

Since photomicrographs are frequently unsatisfactory for demonstrating minute structure it may be necessary to make drawings in order to properly represent the observed structure. Conspicuous structures such as cell walls, large vacuoles, and nuclear outlines may be located by the use of the camera lucida, but the smaller, more indistinct structures must be filled in after removal of the camera lucida. In order that the drawing may be sufficiently large it is necessary that a high-powered objective and ocular should be used while drawing with the camera lucida. After removing this apparatus a combination of objective and ocular giving the best definition should be used.

The camera lucida is attached to the microscope and is exactly centered over the ocular by means of the centering screws. The mirror is then turned to such an angle as to reflect the image on drawing paper which is fastened to a drawing board adjacent to the microscope. If the pencil on the paper is indistinct but the object being drawn is clearly seen, more light is admitted to the camera lucida through the lateral glass discs or less light is admitted through the horizontal glass discs. On the other hand, if the pencil on the paper is clearly seen and the object being drawn is indistinct, more light should enter the camera lucida from below and less light should enter through the lateral glass discs. By regulating these discs it is possible to obtain a combination which will permit the observer to see both the pencil and the image of the object to be drawn.

Allen (5) recommends that drawings should be made on three-ply white Bristol board. The outlines are lightly drawn with pencil with the aid of the camera lucida. This apparatus is then removed and the details are drawn with ink. Various strengths of a good quality

of black drawing ink should be used, the darker portions being drawn with concentrated ink and the lighter portions with the more dilute concentrations. The ink is applied with a pen and with a fine-pointed camel's-hair brush.

After the drawings are completed the edges of the Bristol board are removed until only a narrow margin remains around the drawing. In order that the space on the plate which may contain several drawings may be utilized to the best advantage the following procedure is desirable: A large piece of cheap cardboard is cut so that the proportion of the length to width is the same as that of the page of the journal in which the paper is to be published. If the drawings are to be reduced the cardboard so cut should be several times as large as the page of the periodical. A diagonal is then drawn across the resulting rectangular cardboard and lines parallel to the edge and meeting at the diagonal are drawn at frequent intervals, thus producing numerous rectangles of different sizes but each having the proportion of length to width the same as that of the page of the periodical. The drawings are then arranged in the smallest rectangle in which they can be placed. This procedure enables one to estimate the enlargement or reduction that will be made in publishing the plates. The larger the rectangle covered by the drawings the greater will be the reduction or the less will be the enlargement in reproducing the drawings on a page of the periodical. If less reduction is desired it may be necessary to divide the drawings among several plates. After an arrangement has been decided upon, pieces of a good white cardboard are cut into rectangles of the proper size and the corners of the drawings are pasted to these pieces of cardboard. Printed letters or numbers are pasted on the drawings to designate each drawing or a particular part of a drawing. The size of the letters used should depend on the amount of reduction to be made. The letters should be large enough to be legible after the desired reduction.

CHAPTER III

CULTURE METHODS

The growth of pathogenic organisms in artificial culture media offers many advantages over the methods to which we are limited in studying obligate parasites. Among these advantages may be mentioned:

1. Koch's postulates may be readily applied to organisms which can be grown in artificial culture media. These postulates, as is well known, involve the isolation of the causal organism from the diseased plant, the production of the disease by inoculating healthy plants with a pure culture of the organism, and finally the reisolation of the organism from the inoculated plants. In satisfying these postulates it is evident that the pathogenicity of an organism is conclusively demonstrated. The isolation of a pathogenic organism, as required by Koch's postulates, is most easily accomplished by the use of an agar medium favorable for the growth of the organism. The details of this isolation process will be described later.

2. The morphology of organisms which cause necrosis of the host often cannot be satisfactorily studied when such organisms are in the necrotic host tissues. Such tissues are usually dark in color and do not transmit sufficient light for a satisfactory microscopic study of the organism within. This difficulty may be overcome by studying the morphology of the pathogene grown in artificial culture media.

3. Necrotic diseased tissues are usually invaded by secondary invaders soon after they have been killed by the pathogene. In such tissues which may contain several species of similar organisms it may be difficult to determine whether observed stages are part of the life cycle of the pathogene or of secondary invaders. When an organism is isolated as a pure culture in artificial media one may be sure that all stages observed in the culture belong to the life cycle of a single species.

4. Physiological studies of a pathogenic organism often involve growing the organism as a pure culture in artificial media. Organisms growing in a host or in a mixed culture in artificial media are subject

to many uncontrollable factors which may limit the reliability of physiological studies made under such conditions.

CLEANING GLASSWARE

Glassware which has been in contact with paraffin, balsam, or other substances that are soluble in xylol should be washed in xylol. Since xylol is not miscible with water, the xylol treatment must be followed by alcohol before the glassware is washed with soap and water or chromic acid cleaning solution. This cleaning solution is made by dissolving 100 g. potassium dichromate in 1000 cc. of hot water. After the dichromate is dissolved, cool the solution and then slowly add about 500 cc. of concentrated sulfuric acid, stirring as the acid is added. Before glassware is placed in this cleaning solution, it should be cleaned as well as possible with water. Tubes and Petri dishes containing agar should be boiled in water to remove the agar before they are placed in the cleaning solution. The glassware should be left in the cleaning solution until the adhering material is removed. The solution is then poured off or the glassware is removed from the solution by means of forceps or rubber gloves, and is rinsed through several changes of tap water, and finally in distilled water before draining. Care must be exercised in using the cleaning solution, as it quickly decomposes clothing and injures skin with which it comes in contact.

PREPARATION OF CULTURE MEDIA

Fungi and bacteria are cultured in both liquid and solid media. Solid media have an advantage in being more easily handled without danger of spilling and with less danger of contamination than liquid media. They are also essential for use in isolating organisms. Solid media are usually made by dissolving agar in a liquid favorable for the growth of the organism under investigation. In general, fungi grow best in media rich in carbohydrates and having a *p*H of approximately 5 whereas bacteria thrive in a medium rich in proteins and having a *p*H around 7.

HEATING MEDIA

Since carbohydrates and proteins in an acid or alkaline medium are decomposed by heat, these constituents should be heated no more than is necessary to sterilize them. For physiological studies, solutions of the carbohydrates and proteins should be sterilized separately

and should be aseptically added to the other constituents after sterilization. Since it is necessary to heat agar in an Arnold steamer or autoclave for 1 to 2 hours in order to produce a clear medium which can be easily filtered, it is customary to heat the agar in half of the water to be used in the medium and to add the solution containing the other ingredients after the agar has been thoroughly dissolved. In this way a complete solution of the agar is obtained without subjecting the other ingredients to prolonged heating.

Agar media containing considerable amounts of acid or alkali do not solidify satisfactorily. This difficulty may be obviated by aseptically adding the acid or alkaline constituents to the agar after all other constituents have been sterilized and cooled to 50° C.

CLEARING MEDIA

Liquid or agar media may be cleared with albumin if an especially clear medium is desired. The white of an egg or 10 g. of prepared egg albumin in 100 cc. of water is beaten and is quickly stirred into a liter of the medium which has been cooled to 50° C. The beaker containing the mixture is then steamed in the Arnold steamer for 1 hour. Avoid moving the container during the heating process as this breaks the coagulum into small particles which clog the filter. Tie a piece of cheesecloth around the lip of the beaker to catch the coagulum and pour the media into another vessel or directly into a funnel containing a porous filter paper.

In certain cases, clearing with albumin has rendered media unfavorable for the growth of pathogenes. Clearing should therefore be practiced only when a very clear medium is necessary.

FILTRATION

Absorbent cotton is most commonly used as a filter. This may be placed in a Büchner funnel, or over excelsior or a screen in an ordinary funnel, and filtration may be accomplished either with or without suction. In the case of liquid media, filtration through cotton may be followed by filtration through filter paper. Unless the filtering apparatus and the medium are maintained at a high temperature during the filtration of agar media the filter is likely to clog. Hot water is usually poured through the filtering apparatus just before the hot medium is added. This heats the filtering apparatus and thus prevents solidification of the medium. Incomplete solution of the agar and other constituents of the medium is also likely to prevent satis-

factory filtration. Filter papers suitable for filtering agar media are available and can be used satisfactorily if filtration is carried on in the autoclave or steamer.

SEDIMENTATION

Filtration may be preceded by sedimentation to remove the coarser particles. The medium is heated in a beaker in the autoclave or Arnold steamer. When all soluble constituents of the medium are in solution the heat is turned off, the door being left closed so that the medium cools very slowly and thus allows time for coarse material to settle to the bottom of the container. After the medium has gelled, the beaker may be placed in a vessel of hot water until the medium is loose in the beaker. The gelled medium is then removed from the beaker and the coarse sediment is cut away and discarded. The upper part of the medium is remelted and filtered, or if sufficiently clear may be used without filtering.

HYDROGEN ION CONCENTRATION

According to present theory, a molecule of a compound is made up of an equal number of protons and electrons. If molecules of HCl are placed in water most of these molecules are ionized (dissociated) to form hydrogen ions (H^+) and chloride ions (Cl^-), the hydrogen atom of the HCl molecule giving up its electron to the chlorine atom. Thus the hydrogen ion has one less electron than it has protons and the chloride ion has one more electron than it has protons. Similarly, if molecules of NaOH are added to water most of them are ionized to form sodium ions (Na^+) and hydroxyl ions (OH^-), the sodium atom of the NaOH molecule giving up one of its electrons to the OH.

In pure water most of the water molecules are in the un-ionized state, and if H^+ and OH^- are added to water they have a strong tendency to unite to form more water molecules (HOH). If acetic or other organic acids are added to water only a small proportion of the acid molecules become ionized, most of them remaining in the molecular state. Acids or bases in which most of the molecules become ionized are said to be strong acids or bases; those in which most of the molecules remain in the molecular state are called weak acids or bases.

In culture media the concentration of hydrogen ions and hydroxyl ions is usually low and cannot be conveniently expressed in mols per liter. Consequently the *p*H method of expression is ordinarily used

in such work. *The pH of a medium is the logarithm of the reciprocal of the hydrogen ion concentration.* The logarithm of a number, as is well known, is the exponent indicating the power to which 10 must be raised in order to equal the number. For example, $10^2=100$. Therefore the log of $100=2$. Similarly, $10^3=1000$ and the log of $1000=3$, etc. If a medium has a H^+ concentration of 1/1000 mol per liter its *p*H is computed as follows: The reciprocal of $1/1000=1000$. The log of $1000=3$. Therefore the *p*H of the medium is 3. Pure water has a *p*H of 7. Its hydrogen ion concentration is computed as follows: $10^7=10{,}000{,}000$. The hydrogen ion concentration is the reciprocal of this number, which is 1/10,000,000 mol per liter. In case the logarithm is not a whole number it may be determined by reference to a logarithm table.

Buffers.—Buffers are substances which prevent a great change in *p*H when strong acids or bases are added. This property of a buffer is due to the tendency of an ion of the buffer to unite with hydrogen ion or hydroxyl ion to form undissociated or slightly dissociated compounds. For example, Na_2HPO_4 is a good buffer because it tends to unite with hydrogen ions, as shown in the following reaction, to form the relatively undissociated NaH_2PO_4.

$$Na_2HPO_4 + HCl \rightarrow NaH_2PO_4 + NaCl$$

The same compound may react with a base according to the following reaction to form water which is only slightly dissociated.

$$Na_2HPO_4 + NaOH \rightarrow Na_3PO_4 + H_2O$$

Thus the presence of Na_2HPO_4 in a culture medium would tend to prevent excessive changes in the *p*H of the medium. It is often desirable to have a well-buffered culture medium since certain organisms may produce sufficient acid or basic substances to produce a *p*H in the medium which is unfavorable for their growth. Buffered solutions are also used as standards in the colorimetric determination of hydrogen ion concentration, in the study of enzyme action, in electrophoresis of bacteria and viruses, and in various other physiological studies. Among the compounds which are commonly used as buffers are phosphates, carbonates, acetates, citrates, ammonium salts, and amino acids.

Buffered solutions may be diluted with water to a surprising degree without changing the *p*H of the solution appreciably. This property is made use of in colorimetric determination of the *p*H of

colored culture media. By diluting such media until quite clear the color of the medium does not interfere with the color of the indicator. Numerous buffer solutions have been described by various workers. Literature on this subject is discussed by Clark (506) and Buchanan and Fulmer (494*a*).

Indicators.—Most indicators are compounds which form salts with either acids or bases. The salts dissociate and yield at least one colored ion. The degree of dissociation of the indicator is altered by changes in hydrogen and hydroxyl ion concentration, and the concentration of the colored ion is altered accordingly. Thus the concentration of hydrogen ions in a solution may be determined by the color which develops when indicators are added to the solution.

If considerable colloidal material such as protein is present in a solution it may combine with certain indicators and modify the color. High concentrations of salts in a solution may also modify the color of an indicator. Thus considerable errors may occur in determining the *p*H of a solution by colorimetric methods. It is usually best to use both colorimetric and electrometric methods in determining the *p*H of a solution.

The first step in the determination of the *p*H of a medium by colorimetric methods is the preparation of solutions of indicators. Stock solutions of the indicators should be made up according to the method described by Clark (506) on p. 91. For use the stock solutions are diluted to 250 cc. by the addition of distilled water. Five drops of the diluted indicator solutions are used for each 10 cc. of solution to be tested.

Colorimetric Determination of *p*H.—Two cubic centimeters of media, 8 cc. of distilled water, and 5 drops of the diluted indicator solution are added to a clear test tube having an inside diameter of about 16 mm. After these ingredients are mixed the color of the solution is compared with the colors in Clark's (506) color chart. If it is found that the *p*H is beyond the range of the indicator used, other indicators should be tried until one is found which has the *p*H of the solution near the middle of its range. The *p*H of the solution is then that *p*H which is represented by a color in the color chart having the same shade as the solution being tested. If greater accuracy is desired electrometric methods or the colorimetric methods described by Clark (506) or Fawcett and Acree (525) may be used.

Adjusting the *p*H of Culture Media.—An approximate 4% solution of NaOH is made up and part of this solution is then diluted to produce an exact 1/100 dilution. Twenty-five drops of an indicator

solution having the desired *p*H near the middle of its range, 40 cc. of distilled water, and exactly 10 cc. of the medium are added to a 250-cc. flask. The diluted NaOH solution in a burette is then slowly added to the solution in the flask until the color approaches that of the color in the color chart which represents the desired *p*H. When near the end point of the titration some of the colored solution should be poured into a test tube (having an inside diameter of about 16 mm.) after the addition of each drop of alkali. If the color of the solution in the tube is not the same shade as that in the color chart representing the desired *p*H, the solution in the tube should be returned to the flask and another drop of the alkali added to the solution in the flask. This procedure should be repeated until the color of the solution when placed in the tube is the same shade as that in the chart representing the desired *p*H. The amount of 1/100 dilution of NaOH that was added to the solution is the amount that is necessary to change 10 cc. of the medium to the desired *p*H. The amount of 4% NaOH necessary to change a larger volume of the medium to this *p*H is easily calculated. Such a calculation demonstrates that the volume of 1/100 dilution required to change 10 cc. of the medium to a desired *p*H is the same as the volume of 4% NaOH required to change a liter of the medium to the same *p*H. Before beginning the titration the medium may be made up to about 1025 cc. Two titrations may then be made without reducing the volume of the medium below 1000 cc. Before adding the 4% NaOH to the liter of medium the latter should be measured very accurately with a 1000-cc. volumetric flask. The 10 cc. of medium used during titration should be accurately measured with a 10-cc. pipette and the 4% NaOH to be added to the medium should be measured with a graduated pipette or burette.

If it is desired to reduce the *p*H of the medium, 4% HCl (sp. gr. approximately 1.8) and a 1/100 dilution of this solution may be used in place of the corresponding solutions of NaOH.

In testing or adjusting the *p*H of media containing agar it is necessary that the medium and the water in which it is diluted be maintained at a temperature above 50° C. in order to prevent gelation of the medium.

Since sterilization frequently changes the *p*H of a medium and, in the case of acid or alkaline agar or gelatin media, may prevent gelation, it is often desirable first to sterilize the medium and then to adjust the *p*H aseptically. All glassware with which the sterilized medium has contact should be sterile, and transference of the medium

from one receptable to another should be carried out under a transfer hood.

Electrometric Determination of H.—If a platinum electrode which has been saturated with hydrogen gas is placed in a solution containing hydrogen ions the following reversible reaction occurs at the surface of the electrode.

$$(2H^+) + (2^-) \rightleftarrows H_2$$

The potential of such an electrode is a measure of the tendency for electrons to be given off through the electrode. If the concentration of hydrogen ions is decreased, the above reaction tends to go toward the left and consequently the tendency to give off electrons is greater. The potential of the electrode would therefore be higher with decreased hydrogen ion concentration. By determining the potential of a hydrogen electrode placed in a solution it is possible to estimate the concentration of hydrogen ions in the solution.

In order to determine the potential of a hydrogen electrode it is necessary to attach it to a calomel electrode having a constant known potential. The two electrodes together form a cell or battery which is capable of producing a current. The E.M.F. produced by such a cell is substituted in the following equation to determine the *p*H of a solution.

$$pH = \frac{\text{E.M.F. (observed)} - E}{0.0001983\ T} \qquad (1)$$

T = absolute temperature.

E = potential difference between mercury of calomel electrode and the platinum of a normal hydrogen electrode.

Following are values for E at different temperatures to be used with the saturated calomel electrode:

Temperature (Centigrade)	E
20	0.2488
21	0.2482
22	0.2475
23	0.2468
24	0.2463
25	0.2458

Leeds and Northrup manufacture a very inexpensive calculator for the rapid circulation of *p*H from E.M.F.

The apparatus which is diagrammatically illustrated in Fig. 1 is used for determining the E.M.F. of the hydrogen-calomel cell. As is well known, the drop in potential along a wire of uniform diameter is uniform. Thus, in the 3-volt circuit *ABCD* in which *AB* and *CD* are large wire having negligible resistance, the potential difference between 1.0 and *C* will be 1 volt because the resistance between 1.0 and *C* is $\frac{1}{3}$ of that between *C* and 3.0. Since the potential difference between the electrodes of a dry cell or a series of dry cells (*DA*) may vary with the age of the cells, it is necessary to calibrate the points on the potentiometer (*CE*) before using the apparatus. This is accomplished by connecting the standard cell into the circuit by means of the switches *F* and *G*. This standard cell has a known voltage which is marked on the cell. If the voltage of the standard cell is 1.0188 the contact on the potentiometer (*CE*) is moved to 1.0188 and the contact *K* is closed for an instant. If the null point galvanometer at *L* shows no deflection, this indicates that no current is passing through the galvanometer and that the potential difference between *C* and 1.0188 on *CE* is equal to the potential difference between the electrodes of the standard cell. If deflection of the galvanometer (*L*) is noted when the key (*K*) is pressed, the contact (*J*) should be moved on the resistance (*BE*) until no deflection of the galvanometer occurs when the key (*K*) is closed for an instant. This completes the calibration of the potentiometer (*CE*) and insures that the potential along *CE* is as designated on the scale. That is, the potential difference between *C* and the point marked 1 is actually 1 volt, that between *C* and the point marked 1.2 is 1.2 volts, etc.

In order to determine the potential difference between the terminals *M* and *N* of the hydrogen-calomel cell we now disconnect the standard cell and connect in the hydrogen-calomel cell by means of the switches at *O* and *P*. The key *K* is then pressed for an instant. If deflection of the galvanometer *L* occurs, the contact *H* is moved along the resistance *CE* until no deflection of the galvanometer occurs. When this point is reached the potential difference between *H* and *C* is just equal to that between *M* and *N*, and the potential tending to force electrons up from *H* through the hydrogen-calomel cell is just equal to the potential tending to force electrons down from *M* through *HC* to *N*. Since these two opposite pressures just balance each other no current flows through the galvanometer at *L*.

The reading on the potentiometer *CE* gives the potential difference between *H* and *C*. This potential difference is equal to the potential difference between *M* and *N*, which is the desired E.M.F.

of the calomel-hydrogen cell. This value is substituted in equation (1) and the pH is determined by solving the equation.

The calomel electrode (Q) is made up of a vessel containing a layer of very pure mercury covered with a layer of pure calomel and over this is a saturated solution of KCl which has been saturated with calomel. The vessel R contains a saturated solution of KCl. The tube S contains an agar gel made by heating 3 g. of agar in 100 cc. of water and then adding 40 g. of KCl. The vessel (T) contains the solution to be tested. The platinum electrode dips into this vessel and hydrogen gas is allowed to bubble up over the elec-

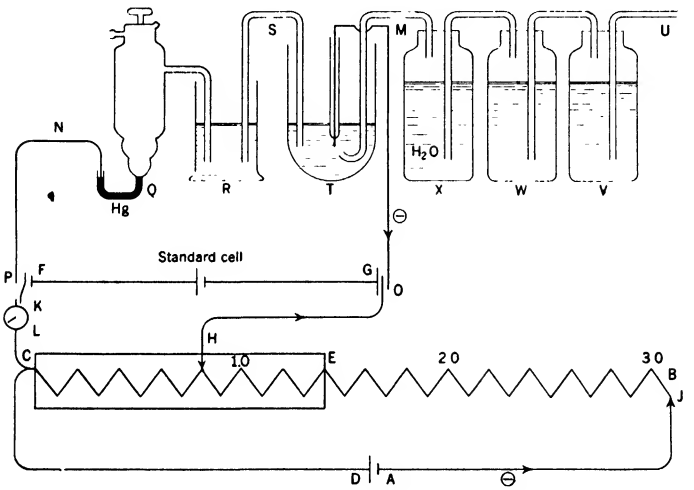


FIGURE 1.—Diagram of apparatus for the electrometric determination of hydrogen ion concentration.

trode. The hydrogen gas is released from the tank U and passes through alkaline pyrogallol solution in bottles W and V to remove any oxygen which may be present in the solution. It then passes through water in bottle X and finally passes around the electrode in vessel T. The pyrogallol solution in bottles W and V is made up by dissolving 10 g. of pyrogallol (pyrogallic acid) in 100 cc. KOH (sp. gr. 1.55). This solution is said to be most satisfactory after standing in stoppered bottles for 2 weeks.

When not in use the electrode may be kept in distilled water. If the electrode requires cleaning it should be placed in concentrated

nitric acid and should be thoroughly rinsed with distilled water before using. Should the electrode continue to give unsatisfactory results it should be placed in aqua regia until bright and should then be recoated with platinum black.

To recoat electrodes with platinum black, connect each electrode with one of the terminals of a 4-volt storage battery or a series of dry cells and place the two electrodes in a 3% solution of pure chloroplatinic acid. Reverse the current every ½ minute during about 3 minutes or until the electrodes are just completely covered by platinum black. Remove the electrodes and wash thoroughly in distilled water. The electrodes are then placed in dilute H_2SO_4 and a current is passed through them as before, the current being reversed every 5 minutes for about ½ hour. The electrode which is connected with the negative terminal of the battery during the last 5 minutes of the process will be found to be most satisfactory since the hydrogen bubbles formed on this electrode tend to carry away all oxygen and to saturate the electrode with hydrogen.

Errors in *p*H determination may be brought about by arsenic or protein deposits on the electrode, and certain electrode "poisons" such as mercury, free ammonia, and H_2S. Strong oxidizing agents also produce erroneous *p*H determinations. Electrometric determinations should always be compared with colorimetric determinations on the same solution.

In order to insure that the apparatus is in order it should be tested at frequent intervals by determining the *p*H of an *M*/20 solution of potassium acid phthalate. According to Clark (506) this solution should have a *p*H around 3.97.

STERILIZATION

Glassware such as Petri dishes and pipettes are usually sterilized by hot air; steam is used for sterilizing the various media used for culturing fungi and bacteria.

Hot-air Sterilization.—A temperature of 150 to 160° C. for at least an hour is necessary for sterilization in hot air. Petri dishes may be wrapped in paper before being placed in the oven in order to prevent infection between the time of sterilization and the time when they are used. If the dishes are to be used immediately this precaution is unnecessary. Dry flasks or tubes should be plugged with cotton before sterilization. Pipettes should be dry and should have a

cotton plug in the upper end before being placed in the pipette can. They are left in the can during sterilization and are not removed until used.

The oven door should not be opened until after completion of sterilization and after cooling, since rapid cooling is likely to produce uneven contraction and a consequent cracking of the glassware.

Steam Sterilization.—Steam, at a pressure of 15 lb., kills all vegetative forms and most spores after a period of 20 minutes. Media which are decomposed by this treatment should be sterilized at 8 lb. for 30 minutes. If this treatment is insufficient for sterilization it may be necessary to repeat the sterilization on three successive days. Resistant spores which are not killed by the first sterilization germinate and produce a vegetative form which is killed by the later sterilizations. The periods given above are for liquid media contained in tubes. Media contained in flasks should be heated longer, since a longer time is required to bring the inner portions to the required temperature. Agar media should be heated for the above periods after they have been liquefied by heating.

Operating Autoclave.—It is necessary that the air in the autoclave should be displaced by steam in order that all parts may be thoroughly heated. This is accomplished by allowing the air to escape through an outlet cock as the steam is introduced into the autoclave. The displacement may be considered complete when steam is rapidly expelled through the cock. After sterilization the steam pressure should be allowed to decrease very slowly to avoid boiling of the medium and the consequent wetting and blowing out of cotton plugs which results when the steam pressure decreases rapidly.

AGAR MEDIA

The following agar media are among those which have been found satisfactory for various plant pathogenes.

Potato-Dextrose-Peptone Agar.—This medium is very satisfactory for the growth of many pathogenic fungi and bacteria.

Solution A=20 g. chopped agar dissolved in 500 cc. distilled water.

Solution B=Potato extract prepared by steaming 200 g. of sliced potatoes in 500 cc. distilled water for ½ hour; 20 g. dextrose and 10 g. peptone are then dissolved in this extract.

Solutions A and B are then mixed and heated in steamer until hot enough for satisfactory filtration or sedimentation.

Many workers omit the peptone when culturing fungi.

Thaxter's Hard Potato Agar.—Bachman (9) has found this medium suitable for yeasts, and other workers have found it a favorable medium for smuts and various other fungi. This medium, as described by Bachman, consists of potato broth, 3% agar, and 2% dextrose.

Canned Corn Agar.—Dr. J. T. Barrett reports that an agar made from canned sweet corn is favorable for the reproductive stages of certain Phycomycetes.

Oat Agar.—Several workers have reported oat agar to be favorable for the development of oospores by species of Phytophthora. Keitt (559) gives the following directions for preparing this media for fungi:

Solution *A*. Add 50 g. of rolled oats to 500 cc. distilled water and heat with stirring over free flame to 70° C. Remove container from flame, wrap with heavy paper and set aside for 1 hour. Filter through gauze and bring filtrate up to 500 cc. by adding more water.

Solution *B*. Add 17 g. of agar to 500 cc. distilled water and cook in steamer until agar is dissolved. Mix solution *A* with solution *B*, heat, and filter through cotton. Tube, and autoclave at 7-10 lb. pressure for 30 minutes.

Prune Agar.—Bacteria do not grow well on this medium but it is a favorable medium for many fungi.

Solution *A*=25 g. agar dissolved in 500 cc. distilled water.

Solution *B*=Prune extract made by steaming 40 g. of dried prunes in 500 cc. distilled water for 1 hour.

Add solution *B* to solution *A*, filter, tube, and sterilize for 15 minutes at 10 lb. pressure.

Pea Agar.—Gwynne-Vaughan and Barnes (539) give the following directions for preparing this medium: "400 dried peas are boiled for an hour, the liquid is poured off and made up to 1000 cc., and 25 g. of agar are added."

Shear's Corn Meal Agar.—To 20 g. of corn meal add 1 liter of water. Keep in a water bath for 1 hour at a temperature of 58° C. Filter through gauze, add 1.5% of agar flour, and steam for 1.5 hours. Filter and tube. Autoclave for 15 minutes at 15 lb. pressure. Dr. Lee Bonar reports that certain organisms, which become sterile when stored on most media, continue to fruit when stored on this medium.

Bonar's Modification of Leonian's (571) Agar Medium.—This is an unusually favorable medium for the growth and reproduction of many fungi and may also be used for bacteria. The peptone appears to be unnecessary for most fungi.

Dihydrogen potassium phosphate	1.2 g.
Magnesium sulfate	0.6 g.
Peptone	0.6 g.
Maltose (or glucose)	6 g.
Malt extract	6 g.
Distilled water	1000 cc.
Agar	1.5–2%

The agar should first be dissolved by heating in 500 cc. of the water. The other constituents should be dissolved by heating in the remaining 500 cc. of water. The two solutions are then mixed and filtered. Sterilize 15 minutes at 10 lb. pressure.

Blakeslee's Agar for Mucors.—According to Dr. Lee Bonar, this medium is very favorable for the mycelial growth of most fungi.

Dry malt extract	20 g.
Dextrose	20 g.
Peptone	1 g.
Agar	20 g.
Distilled water	1000 cc.

Malt Agar.—Piefer, Humphrey, and Acree (604) find that an agar medium made of 25 g. malt extract and 20 g. of agar in a liter of distilled water is very favorable for the growth of wood-destroying fungi.

Grated Carrot Agar.—Johann (556) reports this agar to be favorable for the production of oospores by certain species of Pythium. Grated carrots are placed in the bottom of a test tube to a depth of about 2.5 cm.; 10 cc. of 1.7% agar is then poured into the tube. After plugging with cotton the tubes are autoclaved 30 minutes at 15 lb. pressure. Shredded fresh string beans were found to be equally satisfactory when used in the same way.

Other Plant Agars.—Agar media may be made from many other plants. The hot plant decoction is simply mixed with the agar solution and the mixture is then filtered, tubed, and sterilized. About 50 g. of dry weight of the plant material is commonly used to make up a liter of the medium.

Beef Extract Agar.—The American Public Health Association (481*a*) recommends the following formula for beef-extract agar:

Agar	1.5%
Beef extract	0.3%
Peptone	0.5%
Distilled water	1000 cc.

The Association further recommends that agar should be soaked and washed before using to remove soluble compounds, that white of egg should not be used for clarification, that the medium be sterilized for 20 minutes after pressure reaches 15 lb., and that the *p*H of the medium should be between 6.2 and 7. The above medium is the standard agar medium for bacteria, being the most commonly used agar medium for bacterial plant pathogenes as well as for other bacteria.

Numerous media for bacteria are described in the manual of the Society of American Bacteriologists (627*a*) and by Smith (624).

Synthetic Agar Media.—Synthetic agars have the advantages described for synthetic liquid media. However, agar contains many compounds which may be present in varying amounts, and consequently synthetic media containing agar may be somewhat variable in composition. The soluble constituents of the agar may be removed by dialysis and the variability of the agar thus decreased.

Synthetic agar media should be used whenever possible, the variability in their composition being much less than that of vegetable agar media.

Barnes' (539) Medium

K_3PO_4	0.1 g.
NH_4NO_3	0.1 g.
KNO_3	0.1 g.
Glucose	0.1 g.
Agar	2.5 g.
Distilled water	100 cc.

The above medium is recommended for many fungi.

Claussen's Medium.—This medium has been recommended by several workers as favorable for the production of ascocarps of various Ascomycetes. Gwyne-Vaughan and Barnes (539) describe the technic for using this medium as follows: "Two dishes are used, half of a small Petri dish being placed within a complete larger one. An agar made up with mineral salts and inulin is placed in the inner dish, while the annular space between the walls of the two dishes is supplied with a medium containing the same salts, but no inulin. The inner dish is inoculated, and fruiting occurs when the mycelium has grown into the outer dish. There must be plenty of space between the rim of the inner dish and the lid of the outer, and the spread of the fungus is facilitated if the inner dish is filled nearly full." The medium contains the following ingredients:

KH_2PO_4	0.05	g
NH_4NO_3	0.05	g.
$MgSO_4 \cdot 7H_2O$	0.02	g.
$Fe_3(PO_4)_2$	0.001	g.
Agar	3	g.
Distilled water	100	cc.
Inulin	2	g.

Medium for Wood-Destroying Fungi.—Piefer, Humphrey, and Acree (604) recommend the following synthetic medium for wood-destroying fungi:

Glucose	40	g.
K_2HPO_4	4	g.
Asparagin	4	g.
$(NH_4)_2HPO_4$	2	g.
$MgSO_4 \cdot 7H_2O$	2	g.
$CaCO_3$	0.25	g.
$CaCl_2$	0.1	g.
Agar	15	g.
Distilled water	1000	cc.

Glucose Agar.—This is a valuable medium in that it supports the growth of many fungi but is not favorable for bacteria. Consequently, it is frequently used in isolation work in which it is desired to separate fungi from bacteria.

Glucose	15	g.
Agar	17	g.
Water	1000	cc.

Radicicola Agar.—Diehl (516) was able to culture *Rhizoctonia crocorum* on this agar medium, the medium being acidulated with acetic acid just before pouring into Petri dishes.

Saccharose	10	g.
K_2HPO_4	1	g.
Agar	20	g.
Water	1000	cc.

Horne and Mitter's (552) Medium.—This medium has been found satisfactory for Fusarium species.

Glucose	2	g.
Potato starch	10	g.
Asparagin	2	g.
K_3PO_4	1.25	g.
$MgSO_4 \cdot 7H_2O$	0.75	g.
Agar	15	g.
Water	1	liter

Coon's (605) Medium for Fusarium Species

Saccharose	7.2	g.
Dextrose	3.6	g.
$MgSO_4$	1.23	g.
Potassium acid phosphate	2.72	g.
KNO_3	2.02	g.
Agar	12	g.
Water	1	liter

To this is added malachite green to make 1:40,000 solution or gentian violet to make 1:26,000 solution.

Kellerman's (560) Medium.—The following medium was found favorable for isolating and distinguishing *Phytomonas tumefaciens,* the colonies of this organism being brightly colored by the dye.

Sugar	10	g.
KH_2PO_4	1	g.
$MgSO_4$	0.2	g.
Congo red	0.1	g.
Agar	15	g.
Water	1000	cc.

Crabill's (510) Medium.—This medium is said to be favorable for several strains of *Phyllosticta pyrina.*

NH_4NO_3	10	g.
K_2HPO_4	5	g.
$MgSO_4$	2.5	g.
Sucrose	50	g.
Agar	20	g.
Water	1000	cc.

Other Synthetic Agar Media.—Czapek's liquid synthetic media for fungi and bacteria described under "Liquid Media" may be made into satisfactory agar media by the addition of 15 to 20 g. of agar.

Smith (624) has described numerous synthetic media for bacteria.

Water Agar.—Brown (493) recommends a water agar made of 15 g. of agar in a liter of water for the separation of Saprolegnia, Pythium, Fusarium, and other fungi from bacteria.

Keitt (558) uses a water agar made from 25 g. of agar in a liter of distilled water. The agar is heated in the water until all the agar is liquefied. The medium is then cleared with albumin and filtered through cotton. This filtration is followed by filtration through macerated filter paper in a Büchner funnel, tubing, and sterilization for 20 minutes at 8 lb. pressure. This medium furnishes a very clear medium favorable for single spore isolations. A clear liquid medium

favorable for the germination of spores should be added to the water agar just before pouring into Petri dishes.

PLANT TISSUES

Sterile plant tissues are often very favorable media for pathogenic organisms, fungi frequently sporulating more readily on fragments of plant tissues than on agar or liquid media. Such tissues may be sterilized by heat, or by surface sterilization with disinfectants followed by the aseptic removal of the outer tissues. The latter method of preparing sterile tissues eliminates the changes that may be produced in the tissues by heating. Tissues of potato, carrot, beet, pea, bean, clover, and various kinds of wood are among those commonly used. Dr. J. T. Barrett finds that bean pods are particularly favorable for Aspergillus and Penicillium species. Steam-sterilized plant tissues are very favorable media for Phycomycetes if the tissues are kept moist by a little water in the bottom of the tube.

LIQUID MEDIA

Beef-extract Broth.—This is the standard liquid medium for bacteria. The Society of American Bacteriologists (627*a*) recommends that the medium should have a *p*H between 6.6 and 7 and should have the following composition.

Beef extract	3 g.
Peptone	5 g.
Distilled water	1000 cc.

Medium for Smuts.—Stakman and Christensen (628) recommend a solution of 2% dextrose and 1% malt syrup for the growth of *Ustilago zeae*. Potato-dextrose agar was also used for the growth of this organism.

Plant Extracts.—Extracts of various fruits and vegetables are satisfactory media for plant pathogenes. Prune decoction has been found to be especially favorable as a medium for spore germination. Among the decoctions most commonly used are string bean, lima bean, pea, corn meal, rice, potato, malt, and beet root. Duggar (520) recommends the use of about 50 g. of dry weight of the plant material for every 1000 cc. of water used. On this basis one should use about 490 g. of beet root, 400 g. of string beans, or 120 g. of dried prunes per liter of solution.

If it is desired to sterilize plant extracts without changing the composition by heating, the juice from the crushed tissues should be centrifuged or filtered through sand, macerated filter paper, or some other filter to remove the coarser material and should then be passed through a sterile Seitz Germicide Filter or a Berkefeld "V" filter. The former has the advantage of allowing the extract to filter more rapidly than the Berkefeld type of filter. The apparatus developed by Smith (627) should be used to prevent contamination of the medium during transfer to sterile tubes or flasks. The upper part of this apparatus consists of a glass chamber having one connection for rubber tubing on top, one on the side, and another on the bottom. The upper connection is fastened to a thick-walled rubber tube leading to a water trap which in turn is connected with a suction pump. The side connection is fastened to the filter. A short thin-walled rubber tube leading to a glass tube for filling sterile flasks or tubes is attached to the lower connection. This glass tube is surrounded by a glass hood which prevents infected particles of dust from falling into sterile flasks or tubes while they are being filled. The passage of the liquid from the glass chamber to the flasks or tubes is regulated by a pinch cock on the rubber tube connecting the chamber with the glass tube for filling sterile flasks or tubes. A photograph of the apparatus is shown in Fig. 2.

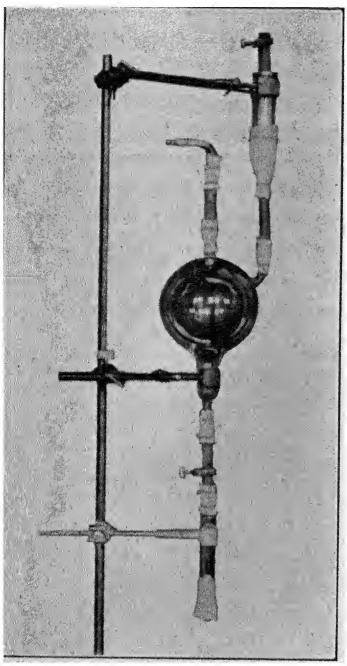


FIGURE 2.—Smith's apparatus for the aseptic filtration of culture media or viruses.

Before sterilization the apparatus is connected with the filter. The lower end of the hood and the connection leading to the water trap are then plugged with cotton, and all joints are wrapped with cotton to prevent contamination by air-borne spores which may be sucked into the apparatus during filtration. After sterilization the rubber tube attached to the lower connection of the chamber is closed by

tightening the screw on the pinch cock, the liquid is poured into the filter, and the suction tube from the water trap is attached to the upper connection on the glass chamber. When the suction is applied, the liquid is drawn through the filter into the chamber until the desired amount of liquid has been filtered. The suction pump is then turned off, the tube from the pump is disconnected from the chamber, and the sterile liquid is allowed to run into sterile flasks or tubes.

Synthetic Liquid Media.—Media made from vegetable decoctions are quite variable in composition, and consequently workers have turned to synthetic media made of pure compounds. Such media should be used when satisfactory growth of the organism under investigation can be obtained. For physiological studies in which the influence of various factors on the organism is under investigation it is quite essential that the medium in which the organism is grown should have a constant composition. Any observations which may be made on the morphology or behavior of an organism grown in synthetic media are especially valuable because the environmental conditions favoring the observed morphology or behavior can be exactly duplicated.

Richard's Solution.—Numerous workers have used this medium for fungi:

KNO_3	10	g.
KH_2PO_4	5	g.
$MgSO_4$	2.5	g.
$FeCl_3$	0.02	g.
Cane sugar	50	g.
Distilled water	1000	cc.

Czapek's Culture Solution for Fungi

$MgSO_4$	0.5	g.
KH_2PO_4	1	g.
KCl	0.5	g.
$FeSO_4$	0.01	g.
$NaNO_3$	2	g.
Sucrose	30	g.
Distilled water	1000	cc.

In order to avoid precipitation, each of the ingredients should be dissolved in 10 cc. or more of distilled water and these solutions then added to enough water to make 1 liter of culture solution. The use of K_2HPO_4 instead of KH_2PO_4 produced a higher *p*H, and is therefore desirable when bacteria are to be cultured.

Tubeuf's Culture Medium.—This medium is said to be satisfactory for the dry rot fungus, *Merulius lacrymans:*

NH_4NO_3	10 g.
K_3PO_4	5 g.
$MgSO_4$	1 g.
Lactic acid	2 g.
Distilled water	1000 cc.

Other Synthetic Media.—It is probable that some of the synthetic agar media described under "Agar Media" would be valuable as liquid media, the agar being omitted in such cases. Smith (624) has given formulæ of numerous synthetic media for bacteria.

GROWTH STIMULANTS

Thompson (636) reports that an unheated extract from potato tubers added to nutrient broth stimulates the growth of certain bacteria which will not grow on the broth alone. One hundred and fifty grams of grated potato tissue are added to 200 cc. distilled water. After shaking 10 minutes, strain and sterilize by filtration; 2 cc. of this extract is added to 6 to 7 cc. of nutrient broth. Extracts of yeast have also been found to stimulate the growth of certain organisms.

ISOLATION METHODS

Diseased plant tissues, or spores, bacteria, or mycelium from such tissues, may be distributed in an agar medium in such a manner that isolated macroscopic colonies of the pathogene develop in the medium. A portion of these isolated colonies may then be transferred to sterile media and the isolated species or strain may be used for inoculating plants or for other experiments in which a pure culture of the organism is needed. The methods commonly used in the isolation of a pathogene are described in the following paragraphs.

PREPARATION OF TRANSFER HOOD

The atmosphere of laboratories often contains many spores which may contaminate plates of agar media poured in such laboratories. Consequently a transfer hood or culture chamber should be used whenever available. Before beginning work the atmosphere of the transfer hood should be sprayed down with a thymol solution. The solution is made by adding 1 cc. of a 10% solution of thymol in 95% alcohol to

100 cc. of water. The surface of the table which is under the hood should be wiped with a small cloth, moistened with the same solution.

SELECTION OF TISSUES FOR PLATING

Since tissues which have been infected for some time usually contain secondary invaders it is usually desirable to select recently infected tissues for isolation work. The advancing margin of necrotic tissues is often free from secondary invaders and may therefore be very satisfactory for plating. In the case of pathogenic fungi, which bear spores while growing on the host, it is often desirable to obtain a suspension of the spores in sterile water and then use this suspension of spores for obtaining a pure culture of the organism.

DISINFECTING THE SURFACE OF DISEASED TISSUES

The surface of diseased tissues is often infected by secondary invaders which quickly overgrow an agar medium if introduced with the pathogene. These secondary invaders may sometimes be killed by disinfectants without injuring the pathogene in the inner tissues. Most of the surface disinfectants in common use do not wet the surface of the tissues uniformly. In such cases the tissues should be immersed in 95% alcohol for about 3 seconds before being treated with a disinfectant.

For thin structures such as leaves, the time of treatment with a disinfectant must be short to prevent killing the pathogene. Smith (624) recommends the use of $HgCl_2$, 1:1000, for 15 to 45 seconds for bacterial leaf spots. Other disinfectants commonly used with various tissues are formalin diluted 1 to 250, or a 50% solution of H_2O_2. These may be used for 15 seconds to 5 minutes or more, depending on the thickness of the diseased tissues and the distribution of the pathogene in the tissues.

After the tissues have been treated with a disinfectant they should be washed for about 5 minutes in each of three changes of sterile distilled water to remove the disinfectant. In the case of thick, fleshy tissues the surface may be disinfected with 70% ethyl alcohol or with methyl alcohol and the alcohol may then be removed by flaming the surface. The outer tissues are then cut away to expose the inner tissues containing the pathogene.

If the tissues are thin and there is consequently danger of killing the pathogene with the disinfectant, or if the infection is very recent and the surface tissues are relatively free from secondary invaders,

it may be undesirable or unnecessary to use disinfectants. Washing such tissues through several changes of sterile distilled water is frequently the most satisfactory method for removing surface contamination. If all available tissues are invaded by secondary invaders which cannot be easily separated from the pathogene it may be necessary to inoculate living susceptible tissues with the diseased tissues and then isolate the pathogene from the inoculated tissues.

SEPARATING TISSUES INTO SMALL PARTICLES

After secondary invaders have been eliminated as far as possible by disinfection of surface tissues it is usually desirable to break the tissues into small particles with the hope that some of the small particles of tissue may contain only a single pathogenic species or strain. This may be accomplished by transferring pieces of diseased tissue to a test tube containing about 2 cc. of sterile distilled water. The tissue may then be crushed into small pieces by an iron rod or scalpel which has been in 95% alcohol for about 3 minutes and has then been lightly flamed to remove the alcohol.

In the case of hosts on which the pathogene bears spores a suspension of spores may be obtained by shaking pieces of the diseased tissue in a tube of sterile distilled water. If sufficient spores are not obtained by this method the fruiting bodies may be cut through or scraped with a sterile scalpel to liberate the spores. The concentration of spores in the suspension is satisfactory when a single loop of the suspension placed on a slide contains approximately the number of spores to be distributed in the agar medium in a single Petri dish. Some workers find that in the case of pycnidia or acervuli fewer contaminations result when the fruiting bodies are allowed to discharge their spores into the water than when they are cut or scraped with a scalpel.

SELECTION OF AGAR MEDIUM FOR PLATING

In general, bacteria grow well on a medium rich in proteins and having a *p*H around 7, whereas fungi usually thrive in a medium rich in carbohydrates and having a *p*H around 5. Beef-extract agar or potato-dextrose-peptone agar is usually very satisfactory for isolating bacteria. If the disease is caused by a fungus and the diseased tissues are free from bacteria, any of the vegetable agar media may be satisfactory. In the case of diseased tissues which have been invaded by saprophytic bacteria 1 to 2 drops of 25% lactic acid should

be added to a tube of the melted agar medium before pouring the plate in order to inhibit the growth of the bacteria. If the fungus fails to grow on the acidified medium, media low in proteins such as glucose agar, prune agar, or water agar should be tried.

DISTRIBUTION OF PATHOGENE IN CULTURE MEDIUM

It is desirable that the particles of diseased tissue, the bacteria, or spores should be sufficiently separated in the agar medium that the resulting colonies are at least ½ inch apart. If the colonies are much closer than this they may become mixed, thus adding to the difficulty of isolating the different species or strains which may be present in the plate. The melted agar medium should always be cooled to 45° C. before the pathogene is added. Higher temperatures may kill the pathogene.

A loop of spores in water may be added to each tube of melted agar before the agar is poured in a Petri dish. The spores in the water should have been previously diluted until the desired number are contained in a loop of the spore suspension. Bacteria may be sufficiently diluted if 1 to 3 loops of a water suspension of crushed diseased tissue are added to each tube of melted agar medium before pouring plates. To insure sufficient dilution a particle of the diseased tissue is transferred to a second tube of sterile water and is then transferred to a tube of melted agar.

In the case of diseased tissue containing mycelium 3 to 5 particles of the crushed tissue should be transferred to a tube of melted agar to be poured in a Petri dish. In any case the tube should be rapidly rolled between the palms of the hands to insure mixing the bacteria, spores, or particles of diseased material in the agar medium. Some fungi do not grow well if covered with a film of agar. In such cases it is necessary to first pour the agar medium in the plate and then add the particles of diseased tissues to the surface of the gelled agar medium.

POURING PLATES

When the pathogene has been properly distributed in the tube of agar medium held at about 45° C. the cotton plug is removed from the tube, the mouth of the tube is flamed, and the contents of the tube are then poured into a Petri dish. When the agar has gelled the plate is inverted to prevent condensation on the cover. The plates are then wrapped in sterile cloth and are incubated in the inverted position at a temperature of 24° to 28° C.

TRANSFERRING ISOLATED ORGANISMS

As soon as isolated macroscopic colonies develop in the poured plates several of those suspected of being the pathogene should be transferred to agar slants or to other sterile media in tubes or flasks. This is accomplished by transferring a small portion from the edge of the colony by means of a sharp, sterile needle. A needle having the tip bent at a 45° angle is usually most satisfactory since it permits greater accuracy in removing the desired portion of the colony. The peripheral portion of a fungus colony is used for transfers because this portion is less likely to be contaminated by bacteria and is more likely to contain only a single fungus strain or species.

If difficulty is encountered in separating the fungus from bacteria a portion of the fungus colony should be transferred to a tube or Petri dish containing water agar, acidified agar, glucose agar, prune agar, or other agar media which are unfavorable for bacterial growth. Brown (493) finds that in a mixed surface colony containing a fungus and bacteria the fungus grows down into the agar while the bacteria remain on the surface. The fungus may be isolated by inverting a portion of the agar by means of a sterile scalpel and then transferring a small portion of the agar containing the mycelium to an agar slant. This method is applicable only to cases in which the agar was inoculated after gelation, and cannot be used satisfactorily when many submerged bacterial colonies are present.

After an agar slant or an agar medium in a flask has been inoculated the cotton plug should be covered with waxed paper and the paper then fastened in place by a rubber band to delay drying of the medium.

SINGLE SPORE ISOLATIONS

In order to insure that a fungus culture contains only a single species or strain it is often advisable to isolate the mycelium developing from a single spore. Numerous methods have been used for single spore isolations. Among them may be mentioned those of Dunn (521), Edgerton (522), Brown (493), Hansen (546), LaRue (569), Dickenson (515), Roberts (615), Hanna (544) (545), Keitt (558), and Malone (579). Various modifications of the method described by Keitt are probably in most general use.

We have found Ezekiel's (523*a*) modification of Keitt's method to be most satisfactory. Following is a description of this method as we have used it: Pour several plates using a clear agar medium favorable for the growth of the fungus. Invert these plates as soon as

gelation of the agar has occurred and allow them to stand about 20 hours until no drops of liquid are present on the surface of the agar. Make a spore suspension in a clear liquid agar medium containing about 0.1% agar. (Any non-toxic liquid having a low surface tension and therefore unfavorable for spore clumping could probably be used in place of the dilute agar medium.) By means of a nichrome wire having a flattened tip the spore suspension is streaked across the surface of the agar in the Petri dishes. The wire should not be allowed to sink into the agar. The streaks should be parallel and placed about an inch apart, the ends of each streak being marked on the lower side of the Petri dish by means of a wax pencil.

The spores are allowed to germinate until a short germ tube develops. The well-isolated spores are then located by examining the streaks with the microscope. The spatula-shaped tip of the nichrome wire used for streaking should be narrow enough to produce a streak the width of which falls within the field of a 16-mm. objective. When a well-isolated spore is located the ocular is removed, the objective is raised, and a small ink dot is placed over the isolated spore, the tip of the pen being visible through the objective of the microscope.

The tip of a second nichrome wire is flattened and is twisted to form a cylindrical "biscuit cutter" on the end of the wire. By the use of this instrument, a disc of agar, about 5 mm. in diameter, containing the isolated spore is cut. Another wire having a spatula-shaped tip about 3 mm. wide is used to transfer the disc of agar from the Petri dish to an agar slant.

Papers giving methods for isolating single bacteria are cited in the bibliography under "Culture Methods" (531) (483) (484) (515) (579) (596) (600*a*) (649).

STORAGE OF CULTURES

As soon as a culture has made the desired amount of growth it may usually be stored for considerable periods without loss of viability if kept at a temperature around 4° C. Church and Scandiffio (505) found that Phytophthora and Pythium species lived longer at room temperature than at 0° C. or 7° C.

REVIVING OLD CULTURES

Povah (607) reports that old cultures may be revived by pouring a slight amount of a favorable melted agar medium into the tube

containing the old culture. The tube is then slanted and, after the agar has gelled, is incubated at 28° C.

Pouring liquid media over old cultures may also cause them to revive.

GERMINATING SPORES

Numerous methods have been described for germinating spores. Perhaps the simplest, and in many cases the most satisfactory method is the following: Take a piece of filter paper which just covers the bottom of a Petri dish. Cut out the center of the filter paper to produce a ring which covers the peripheral portion of the bottom of the dish but which leaves the central portion uncovered. Put the ring of paper in the dish and sterilize the dish and paper in the hot-air sterilizer. Make a suspension of spores in the water or nutrient solution in which the spores are to be germinated and by means of a wire loop transfer a drop of the spore suspension to the under side of the Petri dish cover to form a hanging drop on the cover. If desired, several hanging drops may be placed on a single cover. The filter paper in the bottom of the dish is wet with sterile distilled water to keep up the humidity in the Petri dish. If it is desired that the composition of the hanging drop shall not change, the filter paper should be moistened with a sterile solution having the same composition as the hanging drop. The dishes are then incubated at the desired temperature until germination has occurred. The hanging drops are studied by placing the Petri dish on the stage of the microscope, the hole in the center of the filter paper allowing the light to pass up through the hanging drops.

Since the Petri dish cover is usually nearly 2 mm. thick one must use an objective having a long working distance. Sufficient magnification may usually be obtained by substituting a 15× or 20× ocular for the ocular commonly used.

Another method which is commonly used was described by Melhus (590*a*) and Mains (578). A circle of filter paper which covers the peripheral portions of the bottom of the Petri dish is prepared and placed in the bottom of a clean Petri dish. An ordinary microscopic slide is suspended in the dish, each end of the slide resting on a short glass slip or piece of glass tubing. The dish containing the slide is then sterilized in a hot-air sterilizer. After the dish and slide have cooled, the filter paper is moistened with water or culture solution to maintain a high atmospheric humidity in the dish. A drop of a spore suspension is then placed on the slide. After incubation at the

desired temperature, the cover is removed from the Petri dish and the drop is examined under the microscope. This method is very satisfactory when one does not object to contamination of the drop during examination with the microscope. In many cases contamination of the drop at this time is not objectionable since the preparations are frequently discarded after a single microscopic examination. The method is advantageous in that a cover glass may be added and the germinated spores may then be studied at high magnification.

Duggar (520) has given a detailed description of the use of the Van Tieghem cell in spore germination. As a nutrient medium, Duggar (519*a*) recommends a decoction of green string beans or sugar beets for germinating most readily culturable fungi and finds that many saprophytic fungi require nutrient solutions for germination. Prune decoction is also often used for spore germination. Many parasitic fungi germinate in water.

Fungi vary greatly regarding the temperature required for best germination. Some fungi such as certain rusts germinate best when the spores are chilled on ice before being placed on drops of water. An abundance of oxygen is necessary for the normal germination of many spores. Mains (578) finds that rust spores should be germinated in hanging drops and should not be allowed to become submerged in water. A thin layer of agar on a slide may also be used for the germination of spores which require an abundance of oxygen.

For testing the fungicidal action of substances Reddick and Wallace (611*a*) sprayed slides with the liquid being tested. After drying, a drop of an aqueous suspension of spores is added and the slide is incubated in a moist chamber. Other workers simply make a suspension of spores in the liquid to be tested and then add a drop of the suspension to a slide. After incubation in a moist chamber the percentage of germination and the growth of the germ tubes are determined to gain evidence regarding the fungicidal action of the substance.

When the structure of the cytoplasm and nuclei of germinating spores is to be studied the method given under "Staining Mycelium or Germinating Spores" should be used.

INOCULATING PLANTS

Plants may be inoculated with fungus spores or bacteria by spraying them with a water suspension of the spores or bacteria. In the case of the rusts some workers dust the plants with spores, the plants being sprayed with water just before or after the dusting. If the

atmospheric humidity is high, powdery mildew spores may be dusted on the host without spraying the plants with water.

Zehner and Humphrey (651) made suspensions of smut spores and rust urediniospores in distilled water. By the use of hypodermic needles these suspensions were injected near the growing tip of the host stem.

If wounding is necessary for infection a sterile needle having absorbent cotton wrapped around it is very useful. The needle is immersed in a suspension of the organism and is then used to pierce the plant, thus leaving a drop of the suspension over the puncture. Water sometimes adheres to the plant better if the bloom is first removed from the leaves by rubbing them with clean moist cloth or cotton. Some organisms such as Botrytis should have a nutrient agar or nutrient solution added with the spores or mycelium when making inoculations.

A bell jar or battery jar should be placed over inoculated plants for 24 to 48 hours after inoculation, the covered plants being kept in the shade to prevent "scalding." Many workers have used various modifications of the iceless refrigerator (554) to maintain a high humidity around plants after inoculation. The iceless refrigerator is simply a cloth-covered frame, the cloth cover being kept moist by allowing the upper edges of the cloth to dip in a pan of water or by a fine spray of water which is directed on the cloth. The top of the refrigerator is usually so constructed that no water drips on the plants, all the water running down the cloth on the side. In certain cases it may be desirable to direct a fine spray of water on the plants.

It is frequently possible to infect detached leaves and to maintain the leaves and parasite in a vigorous condition for some weeks. This technic has been used by Mains, Clinton, and other workers. Waters (639) reviews the literature on this subject and describes the following method for rusts: Remove leaves from the host and immediately place them in water; wash the leaves in running water several minutes and then through several changes of sterile distilled water; spray a suspension of spores on the leaf contained in a Petri dish and add just enough water to the dish to keep the air saturated for 48 hours; a 5 to 7% unsterilized solution of commercial sucrose is then added to the dish and the leaf is floated on this solution. The dishes are stored in diffuse light, the petioles being cut back at intervals to facilitate intake of nutrients. The leaves are washed in sterile distilled water several times per week and the sugar solution is renewed at this time; any contaminations observed on the leaves are also cut off at this

time. Giddings and Leonian (533) were able to grow rust-infected apple leaves in modified Pfeffer's solution plus 0.5% glucose for nearly 5 months.

Roots may be infected by placing inoculated agar in the soil or by mixing inoculated wheat, corn meal, or oats with the soil around the roots.

Failure to infect plants should not be considered as proof that the suspected organism is not pathogenic, since many pathogenes produce infection only under certain conditions of temperature and humidity. Frequently the stage of maturity of the inoculated tissues is important also.

In the case of heterothallic fungi it may be necessary to inoculate with both the plus and minus strains in order to produce typical disease symptoms.

CHAPTER IV

VIRUS STUDIES

The number of diseases which have been shown to be due to viruses has become so great that this class of diseases must now be considered one of the most important with which the phytopathologist has to deal. The methods used in studying the virus diseases are quite different from those used in studying other classes of plant diseases, much of the work involving the use of insects and consequently requiring that the worker have a certain amount of entomological knowledge.

A survey of the literature indicates that all virus diseases may be transmitted by grafting if a union can be obtained between the diseased and healthy tissues. Some of these diseases are also readily transmitted by juice inoculations; others have not been transmitted by this method but are regularily transmitted by certain insects.

VIRUS EXTRACTION

Most virus studies have been made on viruses in juice extracted from the leaves and stems of diseased plants. Severin has recently shown that juice from the roots of beet plants infected with the curly-top virus is infective to hoppers but he was unable to produce infection of hoppers by feeding them juice extracted from leaves of the same plants.

The tissues are usually crushed by the use first of a food chopper and then a mortar and pestle, and the juice is extracted by pressing the pulp in cloth. More juice is obtained if the leaves are frozen before crushing.

It is always advisable to remove as much of the coarser material as possible before passing the juice through a Berkefeld filter. Otherwise the filter quickly becomes clogged and consequently greatly retards the speed of filtration. The juice should first be centrifuged to remove as much of the coarse material as possible. After this treatment the juice should be quite free from coarse colloidal material and will pass through Berkefeld filters. These filters are made in three

grades, each grade having a different size of pore and consequently limiting the size of particles which may pass through the filter. The grade having the largest pores is called the "V," that having medium-sized pores is the "N," and that having the smallest pores is called the "W." The method of using these filters with Smith's filtration apparatus was described earlier under "Plant Extracts." The virus is filtered and transferred to sterile tubes in the same way that plant juices are sterilized by filtration for use as culture media.

Some viruses are inactivated a few hours after extraction from the host. Inoculations should therefore be made immediately after filtration. Long and Olitsky (725*a*) report that inactivation of vaccine virus is delayed if the virus is stored under anaerobic conditions. Adding cysteine hydrochloride to the virus and then sealing the tubes with petrolatum was found to inhibit the inactivation of the virus. The cysteine hydrochloride solution was adjusted to *p*H 7.5 and enough of the solution was then added to the virus to give a 1:2000 dilution of the chemical. Gye and Purdy (688*a*) reported that the addition of small amounts of HCN to Rous sarcoma virus No. 1 inhibited the inactivation of the virus.

INOCULATIONS

Since most virus diseases may be transmitted by certain insects it is usually desirable to protect inoculated as well as check plants from insects by covering them with insect-proof cages. If the plants do not grow well under cages they may be grown in the open, but a sufficient number of check plants as well as inoculated plants should be provided to furnish a basis for estimating the amount of accidental infection.

GRAFTING

The available evidence indicates that all virus diseases may be transmitted by grafting if a satisfactory union between the healthy and diseased plant can be obtained. This method of transmission is therefore the most reliable of the transmission methods if the host plant can be easily grafted. Shrubs or trees may be grafted by budding or by any of the common methods of grafting. Herbaceous plants may be most easily grafted by the use of the approach graft. A strip is cut off on the side of each stem and the cut surfaces are then bound together until union results. The two plants are either grown in the same pot or in adjacent pots.

TRANSMISSION BY INSECTS

Most of the virus diseases have been transmitted by insects, some of them having been transmitted only by a single species. Insects may be collected in the field with an insect net. They are then transferred to an insect cage containing leaves of the host plant or a potted plant upon which they may feed until taken to the greenhouse. When possible, a non-infective strain of insects should be developed by rearing them on some host which is not susceptible to the disease or by placing the nymphs on a healthy plant as soon as they have hatched and before they have had a chance to feed. Some of these non-infective insects should be placed on caged diseased plants for several days and should then be transferred to healthy plants in an attempt to infect the latter. Non-infective insects are placed on other caged healthy plants which are to serve as checks. The insects should be left on the plants for three or more weeks since an "incubation period" in the insects is sometimes necessary before they become infective.

Insects are easily handled by means of a pipette described by Severin and Swezy (770). A 10-cc. pipette is cut off about an inch below the bulb. A small piece of strong thin cloth is then fitted over the cut end of the pipette to prevent escape of the insects, and the end of a rubber tube about 2 feet long is then attached to the cut end of the pipette by placing it over the piece of cloth. By sucking on the end of the rubber tube the insects may be drawn into the pipette, there examined, counted, and then dropped into a cage containing a plant.

Insects may sometimes be infected by allowing them to feed on filtered virus contained in a membranous sac as described by Carter (672). By this method it is possible to study the properties of a virus which is only readily transmitted by insects.

JUICE INOCULATIONS

Some of the virus diseases may be easily transmitted by inoculating wounded tissues of a healthy plant with filtered juice from a diseased plant. Holmes (700) and Samuel (765) have found that rubbing the leaves with a cloth or a flat ground-glass spatula which has been dipped in the virus is the most reliable method of producing infection by juice inoculation.

Numerous papers describing other methods used in virus studies are cited in the Bibliography.

CHAPTER V

MISCELLANEOUS EXPERIMENTAL METHODS

PRESERVING DISEASED MATERIAL FOR MUSEUM SPECIMENS

Copper acetate crystals are added to a 50% solution of acetic acid until no more will dissolve. To 1 part of this solution add 4 parts of water, put the specimen in this diluted solution, and boil over a flame. At this temperature the green of the chlorophyll breaks down and a yellowish-green color appears. As boiling continues the green color of the copper acetate replaces the yellowish-green. The time of boiling may vary from 3 to 15 minutes. When the proper intensity of color is reached the specimen should be removed, washed in tap water, and stored in a solution of 70% alcohol or a solution containing 5 parts of formalin in 100 parts of water. Aside from its expense, the alcohol is probably more satisfactory than formalin since the latter tends to cause the green color to fade and allows tissues to soften.

Other methods for preserving specimens are described by Keefe (852), Hedgcock and Spaulding (835), Nieuwland and Slavin (883), Stover (914), and Woods (939).

TEMPERATURE AND HUMIDITY CONTROL

The methods which have been described for maintaining a constant temperature and humidity are so numerous that they cannot be included here. Apparatus for growing plants at a constant temperature and humidity have been described by numerous workers: (811), (830), (837), (848), (859), (894), (896), (911), (913), and (938). Methods for controlling the humidity in small vessels are described by Wilson (937) and Stevens (630), who use various concentrations of sulfuric acid. Spencer (910*a*) recommends the use of saturated solutions of solid compounds containing an excess of the solid for the same purpose. He includes a table showing the relative humidity produced by a large number of compounds.

Other methods for controlling and recording environmental factors are cited in the Bibliography.

GROWING PLANTS UNDER STERILE CONDITIONS

Numerous methods have been described for sterilizing seeds and growing plants under sterile conditions: (485), (514), (563), (577), (581), (638), (645), and (650).

PART III

CHAPTER VI

INTERPRETATION OF EXPERIMENTAL RESULTS

When possible it is desirable that the results of experiments be subjected to mathematical analysis in order to determine whether they appear to be significant, and if so, the degree of confidence which may be placed in this apparent significance. The plant pathologist often performs experiments which yield quantitative results. For example, he may measure a large number of spores from similar fungi in order to gain evidence as to whether the fungi, being compared, show any distinct differences in spore size. He may compare the weights of a number of plants inoculated with a particular organism with the weights of uninoculated control plants in an attempt to gain evidence as to the pathogenicity of the organism. The results of such experiments should be subjected to statistical analysis.

An attempt is made in the following pages to present methods which are applicable in the analysis of quantitative experimental results.

Suppose that one determines the dry weight of a large number of inoculated plants and the same number of uninoculated plants. The determinations are then paired, the weight of one inoculated plant and one uninoculated plant constituting each pair. The members of each pair should be strictly comparable. For example, if there is no danger of accidental transmission of the disease, the two members of a pair should be grown in adjacent positions in the greenhouse. The difference between the dry weight of the two plants in each pair is determined and the differences are then grouped into classes of equivalent range. For example, all differences between 19 and 21 would be put in the class having a class value of 20, those between 21 and 23 would be put in a class having a class value of 22, etc. The number of differences in each class are then plotted as ordinates against the class values to produce a frequency curve. If the number of differences is large, the curve usually is similar to the normal curve

or curve of error shown in Fig. 3. The mean difference for the entire group would be at M. This mean is determined by adding all the differences and dividing by the number of differences.

If perpendiculars are drawn at M, Q_1, and Q_3 to divide the area under the curve into four equal areas, then the number of differences falling to the left of Q_1 is equal to the number falling between M and Q_1, to the number between M and Q_3, and to the number to the right of Q_3. Therefore if a single difference is chosen at random from the numerous differences the chances are even as to whether this difference will differ from M by more or less than the length of the line MQ_1. In other words, the chances are even as to whether it will fall between Q_1 and Q_3 or outside of this region. The chances are about 30 to 1 that the single difference would not differ from M by more than 3.2 times the length of MQ_1. For example, in Fig. 3

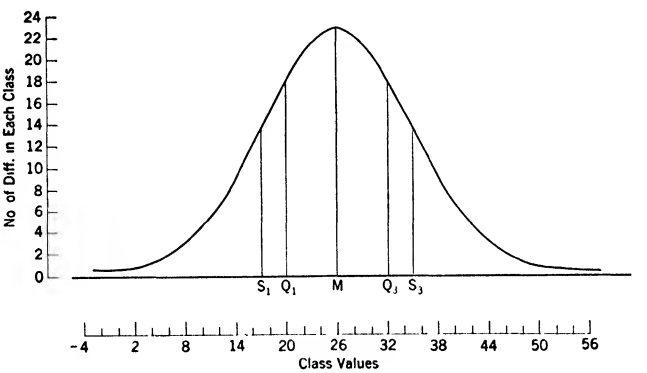


FIGURE 3.—Normal curve or curve of error.

the mean difference $M=26$. The probable error, which is the length of the line MQ_1, is 6.

$$3.2\times 6 = 19.2$$
$$26-19.2 = 6.8$$
$$26+19.2 = 45.2$$

Therefore the chances are approximately 30 to 1 that a single difference chosen at random would fall between 6.8 and 45.2.

The question then arises as to where one should expect the mean M to fall if the experiment were repeated. It is obvious that one should expect less variation in means than in single determinations.

The probable error of the mean is determined by the application of the following equation:

$$\text{Probable error of mean} = \frac{MQ_1}{\sqrt{n}}$$

In this equation, n is the number of differences determined. Suppose that the number of differences determined in preparing Fig. 3 is 81 and $MQ_1=6$. The probable error of the mean would be $6 \div \sqrt{81} = 0.66$. The following calculations are then made in order to determine where the mean would be expected to fall:

$$\begin{aligned} 0.66 \times 3.2 &= 2.1 \\ 26 - 2.1 &= 23.9 \\ 26 + 2.1 &= 28.1 \end{aligned}$$

The odds are therefore approximately 30 to 1 that if the experiment were repeated the mean difference would be between 23.9 and 28.1. The odds that the mean would be greater than zero, or in other words would be positive, would of course be much greater than 30 to 1. Odds of more than 30 to 1 are ordinarily considered conclusive in interpreting biological data. One would therefore conclude that the organism used as inoculum produced a distinct decrease in the dry weight of the plants.

The probable error has been shown to be an indicator of the variation in the results. Another indicator which is used more frequently is the standard deviation MS_1 shown in Fig. 3. The standard deviation is determined by applying the following equation:

$$MS_1 = \sqrt{\frac{\Sigma(d^2)}{n-1}}$$

In this equation, d represents the deviation of each difference from the mean difference. The deviation of each difference is squared and the sum of the squared deviations is represented by $\Sigma(d^2)$; n is the number of differences.

The standard deviation MS_1 is somewhat larger than the probable error MQ_1. The latter may be calculated from the former, since $MQ_1=0.6745\ MS_1$. From this equation it can be calculated that $3.2 \times MQ_1 = 2.16 \times MS_1$. Therefore the odds are approximately 30 to 1 that a single difference chosen at random would not differ from the mean by more than 2.16 times the standard deviation. The standard

deviation of the mean is obtained by applying the following equation:

$$\text{Standard deviation of mean} = \frac{MS_1}{\sqrt{n}}$$

Similarly the odds are approximately 30 to 1 that if an experiment were repeated the mean would not differ from the observed mean by more than 2.16 times the standard deviation of the mean. The above equations for determining probable error and standard deviation, and the odds given above, apply only to the normal curve and are not applicable if the frequency curve has a form which differs greatly from that of the normal curve.

Student (953) has developed a modification of the above method which is more reliable when only a small number of differences is available. It must be observed, however, that no method of statistical treatment can be satisfactory unless sufficient data are available. Because of the great variation usually observed in biological data it is doubtful whether one should attempt to draw conclusions in most biological work unless at least 5 differences are available. Student's method apparently has more application in phytopathological work than have other statistical methods, since it is seldom practicable in such work to obtain a large number of differences. An example of its application follows:

Suppose that one wishes to compare the value of two fungicides in controlling a disease. Seven plants are sprayed with each fungicide and the number of lesions on each plant is recorded. The results are shown in Table 4. The difference in the number of lesions on the plants in each pair is determined. The mean difference and the deviation of each difference from the mean difference is then determined. Values are substituted in the equation for determining the standard deviation and the standard deviation is found to be 5.28. The mean difference is then divided by the standard deviation to give a value of 1.57 for Z. Love (949*a*) gives a table showing the odds for different values of Z. Looking up Z in Love's tables we find odds of 255 to 1 when $Z=1.6$ and $n=7$, and odds of 216 to 1 when $Z=1.55$ and $n=7$. By interpolation, a value of 232 is found as the odds when $Z=1.57$. According to Student (953), these are the odds that the mean of the population of which this experiment is a sample is positive. In other words, if the difference were determined for a very large number of plants the odds are 232 to 1 that the mean difference would be positive. Since odds of more than 30 to 1 are ordinarily considered con-

clusive one would conclude that fungicide *A* is distinctly better than *B* in preventing infection.

TABLE 4

COMPARISON OF FUNGICIDES *A* AND *B* IN CONTROLLING A DISEASE

Pair Number	Number lesions on plant sprayed with fungicide *A*	Number lesions on plant sprayed with fungicide *B*	Difference in number of lesions	*D*	D^2
1	10	25	15	6 7	44.89
2	13	12	−1	−9.3	86.49
3	8	14	6	−2.3	5 29
4	3	15	12	3 7	13.69
5	5	12	7	−1.3	1.69
6	20	27	7	−1.3	1.69
7	6	18	12	3.7	13 69

$M = +8.28$ $\Sigma D^2 = 167.43$.

Standard deviation $= \sqrt{\frac{167.43}{6}} = 5.28$

$Z = \frac{8.28}{5.28} = 1\ 57.$

Odds = 232 to 1.

M = mean difference.

D = deviation of each difference from mean difference.

Other statistical methods are described by Babcock and Clausen (940) and in other works cited in the Bibliography. The determination of the coefficient of correlation, as described by Babcock and Clausen (940), may sometimes be helpful in interpreting the results of phytopathological research.

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